Modeling the Interleukin 2 gene expression in activated T cells

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Abstract

Interleukin 2 (IL-2) is a cytokine expressed in human memory T helper cells and the secretion of IL-2 shapes the magnitude and duration of immune responses. In contrast, human regulatory T-cells commonly do not express IL-2. The diverse effects of IL-2 on protective immune responses as well as peripheral immune tolerance form the basis of the profound role of IL-2 in various diseases. The gene expression of IL-2 is induced by the activation of the T-cell receptor signaling network activating the transcription factors AP-1, NFAT, and NF-κB. These transcription factors are crucial for initiating IL-2 gene expression.

Within my thesis I explore the regulation of IL-2 gene expression in a detailed modeling approach combined with experiments. I compare the regulation in memory effector Th cells (Teff cells) and regulatory T-cells (Treg cells). In particular, I demonstrate that the transcription factor concentrations correlate with number of IL-2 producers but do not affect IL-2 concentration per cell. Thus, I investigate how the transcription factor concentration of c-fos, NFATc2, p65, and p-c-jun affects the frequency of IL-2 producing cells as a proxy for the probability of a cell to produce IL-2. Using the endogenous heterogeneity of transcription factor concentrations, I predict that the number of IL-2 producers is critically dependent on the amount of c-fos in Teff cells. I use my established model to predict how perturbations of c-fos by the specific inhibitor U0126 decrease the frequency of IL-2 producers in Teff cells. This prediction was than validated by experiments. My models furthermore indicate the cooperative behavior of c-fos and NFATc2 on the level of frequency of IL-2 producers in Teff cells.

In Treg cells, my analysis shows that all transcription factors exert a similar sigmoidal effect on the frequency of IL-2 producers. In contrast to the effects seen in Teff cells, all transcription factor have a similar maximal effect on the IL-2 gene expression. I employed an inhibitory model to explore the relation between the Treg cell-specific transcription factor FoxP3 the transcription factors c-fos, NFATc2, p65, and p-c-jun on the frequency of IL-2 producers. This model indicates that FoxP3 counteracts the activating function of NFATc2, AP-1, and also NF-κB.

My results are discussed in the context of kinetic proofreading in an immune response and with respect to their implications for autoimmune diseases, such as systemic lupus erythematosus (SLE). Parts of this thesis have been published in “Stable IL-2 decision making by endogenous c-fos amounts in peripheral memory Th cells.” (Bendfeldt et al., 2012a) and in “IL-2 Expression in Activated Human Memory FOXP3(+) Cells Critically Depends on the Cellular Levels of FOXP3 as Well as of Four Transcription Factors of T Cell Activation.” (Bendfeldt et al., 2012b).

Zusammenfassung

Interleukin 2 (IL-2) ist ein Zytokin, welches in menschlichen Gedächtnis-T-Helfer-Zellen (Teff Zellen) exprimiert und sekretiert wird und damit die Stärke und Dauer der Immunantwort formt. Im Gegensatz dazu wird IL-2 normalerweise nicht in regulatorischen T-Zellen (Treg Zellen) exprimiert, sondern von diesen nur aufgenommen. Die Produktion von IL-2 spielt eine wichtige Rolle bei verschiedenen Krankheiten, was sich durch die vielfältigen Auswirkungen von IL-2 auf die Immunantwort und auch die periphere Immuntoleranz erklärt. Durch die Aktivierung des T-Zellrezeptors werden Signalkaskaden induziert, welche zur Aktivierung der Transkriptionsfaktoren AP-1, NFAT, und NF-κB führen. Diese Transkriptionsfaktoren sind entscheidend für die Genexpression von IL-2.

Im Rahmen meiner Dissertation habe ich die Regulation der IL-2 Genexpression mit Hilfe von Modellierung zusammen mit Experimenten erforscht. Dabei vergleiche ich die transkriptionelle Regulation in Teff Zellen mit der Regulation in Treg Zellen. Insbesondere konnte ich zeigen, dass die endogene Konzentration der Transkriptionsfaktoren sich auf die Anzahl der IL-2 Produzenten auswirkt, aber nicht auf die Konzentration von IL-2 innerhalb einer Zelle. Deshalb untersuche ich im Weiteren, wie sich die Konzentration der Transkriptionsfaktoren c-fos, NFATc2, p65 und p-c-jun auf die Häufigkeit von IL-2 Produzenten auswirkt. Ich nutze die vorhandenen körpereigenen Konzentrationen und kann damit vorhersagen, dass die Zahl der IL-2 Produzenten entscheidend von der c-fos Konzentration in Teff Zellen abhängt. Mit Hilfe des entwickelten Modells kann ich voraussagen, wie der spezifische Inhibitor U0126 die Häufigkeit von IL-2 Produzenten verringert. Diese Vorhersage wurde durch Experimente belegt. Meine Modelle zeigen weiterhin, dass c-fos und NFATc2 die Häufigkeit der IL-2 Produzenten in Teff Zellen kooperativ regulieren.

In Treg-Zellen zeigt meine Analyse, dass alle Transkriptionsfaktoren eine ähnliche sigmoidale Wirkung auf die Häufigkeit der IL-2-Produzenten ausüben. Im Gegensatz zu den Teff Zellen haben alle Transkriptionsfaktor eine ähnliche maximale Wirkung auf Genexpression von IL-2. Mittels eines Inhibitionsmodelles konnte ich zeigen, dass der Treg zellspezifische Transkriptionsfaktor FoxP3 allen aktivierenden Transkriptionsfaktoren entgegenwirkt.

Ich diskutiere meine Ergebnisse im Rahmen der Idee des kinetic proofreadings innerhalb der Immunantwort und welche Auswirkungen das auf Autoimmunerkrankungen, wie zum Beispiel den systemischen Lupus erythematodes (SLE), hat. Teile dieser Dissertation wurden in “Stable IL-2 decision making by endogenous c-fos amounts in peripheral memory Th cells.” (Bendfeldt et al., 2012a) und in “IL-2 Expression in Activated Human Memory FOXP3(+) Cells Critically Depends on the Cellular Levels of FOXP3 as Well as of Four Transcription Factors of T Cell Activation.” (Bendfeldt et al., 2012b) veröffentlicht.

Contents

List of Terms and Acronyms

AICc	Akaike's information criterion, corrected for sample size
AML	acute myeloid leukemia
AP-1	activator protein 1
APC	antigen presenting cell
ARRE-1/2	antigen receptor response element 1 & 2
Bcl10	B-cell lymphoma/leukemia 10
BIC	Bayesian information criterion
bp	base pairs
CARMA-1	caspase recruitment domain-containing MAGUK protein-1
CD28	cluster of differentiation 28
CD28RE	CD28 response element
CD3	cluster of differentiation 3
CD4	cluster of differentiation 4
CD45	cluster of differentiation 45
CD8	cluster of differentiation 8
CRAC	calcium release-activated calcium channel
CREB	cAMP response element-binding protein
CREM	cAMP-responsive element modulator
CsA	cyclosporin A
CTLA-4	cytotoxic T-Lymphocyte antigen 4
DAG	diacylglycerine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

EAE	experimental autoimmune encephalomyelitis
ER	endoplasmatic reticulum
ERK	extracellular-signal-regulated kinase
FACS	fluorescence-activated cell sorting
FI	fluorescence intensity
FITC	fluorescein isothiocyanate
FoxP3	forkhead factor P3
GATA4	GATA binding transcription factor 4
GDP	guanosine diphosphate
GoF	goodness of fit
GRB	growth factor receptor-bound protein
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HMGI(Y)	high-mobility group I(Y)
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IC_{50}	half maximal inhibitory concentration
IFNγ	Interferon γ
IKK	IκB kinase
IL-2	Interleukin 2
IP_3	inositol trisphosphate
ITAM	immunoreceptor tyrosine based activation motif
JNK	c-Jun N-terminal kinase
JNKK	Jun kinase kinase
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1
Mef	myocyte enhancer factor 2
MEK	mitogen-activated extracellular-regulated kinase
MEKK	mitogen-activated extracellular-regulated kinase kinase
memory Th cells	memory T helper cells
MHC	major histocompatibility complex
MM	Michaelis-Menten-like

NF-κB	nuclear factor κ-light-chain enhancer of activated B-cells
NFAT	nuclear factor of activated T-cells
Oct	octamer binding transcription factor
PE	phycoerythrin
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKCθ	protein kinase C θ
PLCγ	phospholipase C γ
PMA	phorbol 12-myristate 13-acetate
Ras	rat sarcoma
RasGEF	Ras-GTP-exchanging factor
RHD	Rel homology domain
RSS	residual sum of squares
SLE	systemic lupus erythematosus
TCR	T-cell receptor
Teff cells	memory effector T helper cells
TF	transcription factor
Tfh cells	follicular B Helper T-cells
Th	T helper
TNF-β	tumor necrosis factor β
Treg cells	regulatory T-cells
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
Zap70	ζ-chain-associated protein kinase 70

1. Introduction

The mammalian immune system consists of the innate and the adaptive immune system, which together constitute an effective defense mechanism against infectious pathogens (Janeway et al., 2002), such as bacteria, fungi, protozoa, worms, viruses, and even infectious proteins called prions (Alberts et al., 2008). The innate immune system is able to distinguish pathogens and the body's own cells effectively creating a first barrier against pathogens. If an infection is not eliminated, the innate immune system triggers activities leading to an adaptive immune answer (Janeway et al., 2002).

One major task of the cells in the adaptive immune system is the production and secretion of effector proteins, in particular cytokines, which function as extracellular messengers. Some effector proteins, such as Interferon-γ and tumor necrosis factor β (TNF-β) activate macrophages, whereas other effector proteins, such as Interleukin 2 (IL-2) and Interleukin 4 activate the proliferation of T-cells. Some of the effector proteins have an activating as well as inhibiting effect on cells of the adaptive immune system. For example, TNF-β not only activates macrophages but also inhibits B-cells and kills T-cells (Janeway et al., 2002). This concerted action of effector proteins leads to a balanced immune answer.

The effector protein IL-2 is a cytokine, which is expressed in and secreted from cells of the adaptive immune system. Initially IL-2 was found to stimulate the immune answer and to play an active role in the defense of an organism against pathogens. In the last years, the focus of research has shifted from immune-stimulating to immune-attenuating effects of IL-2. This lead to a picture of IL-2 playing an important role in balancing the response of the immune system.

In this chapter, I will introduce the cell types of the immune system, in particular T-cells in which IL-2 is expressed (Section 1.1). Furthermore, I will explain the function and importance of IL-2 in balancing the adaptive immune system (Section 1.2). After that, I will describe the activation of T-cells leading to IL-2 gene expression (Section 1.3) and discuss the transcriptional regulation of IL-2 (Section 1.4). In the next section of this chapter, I will introduce flow cytometry as a method of choice for counting, sorting, and characterizing immune cells (Section 1.5). The following section is an overview of modeling approaches to study IL-2

gene expression (Section 1.6). I will conclude this chapter with a description of my research objectives for this thesis (Section 1.7).

1.1. T helper cells and their role in the adaptive immune system

The immune system consists of two parts. The innate immune system is the first barrier defending the body against attacks by pathogens, such as microbes and viruses. The pathogens are first detected by macrophages, neutrophils, and dendritic cells, which take them up and induce phagocytosis. During this process proteins of the pathogen are lyzed. Their fragments are presented at the membrane of dendritic cells to cells of the adaptive immune system. This characteristic of antigen presenting cells (APCs) connects the innate and adaptive immune system (Figure 1.1). The different cell types of the adaptive immune system have evolved specialized defense strategies against different kinds of pathogens. They are called humoral or cell-based immune answer.

B-cells produce and secret antibodies constituting the humoral immunity, which refers to extracellular immune answers. These antibodies bind their corresponding antigens at the pathogen's membrane marking the pathogens for destruction (Alberts et al., 2008). Furthermore, B-cells are able to develop a memory, which enables a faster immune reaction upon a second encounter of the pathogen.

T-cells represent the cell-based part of the adaptive immune system. They can further be separated into cytotoxic T-cells and T helper (Th) cells. Both subtypes express the T-cell receptor (TCR), which is able to recognize antigens presented at the surface of other cells in combination with major histocompatibility complexs (MHCs). Additionally, cytotoxic T-cells express the glycoprotein cluster of differentiation 8 (CD8), that supports binding to antigens presented by complexes of class MHCI. Virally infected cells and tumor cells that present extraneous proteins at their surface by the MHCI are therefore recognized and destroyed by cytotoxic T-cells. In contrast, Th cells express the cluster of differentiation 4 (CD4). It activates the TCR when it binds to an antigen presented by the MHCII. As these are expressed by APCs, Th cells orchestrate the acute immune answer via the secretion of cytokines (extracellular messengers) and the activation of B-cells. Similar to B-cells, Th cells develop a memory leading to a faster immune answer in case of a second contact with the pathogen.

So far, five subtypes of Th cells have been described in the literature (Figure 1.2). These different subtypes differentiate from naive Th cells upon first encounter of an antigen. Each subtype secretes a specific set of cytokines, which influences the other cells of the adaptive

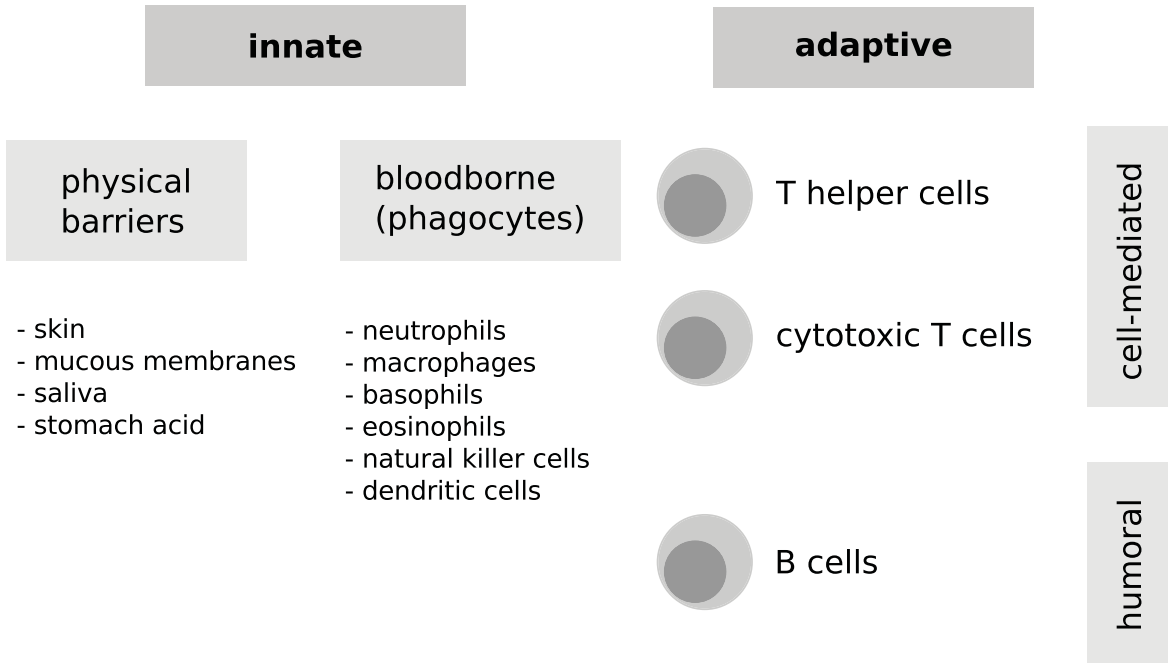

Figure 1.1.: Overview of the innate and adaptive immune system. The innate immune system consists of physical barriers and a blood-borne cell defense, including macrophages, neutrophils, and dendritic cells. Pathogens are lyzed and their fragments, so called antigens, are presented at the membrane of dendritic cells. These antigen presenting cells (APCs) are connecting the innate and adaptive immune system. The adaptive immune system consists of humoral immunity, including B-cells producing and secreting antibodies, and cell-mediated immunity, including different types of T-cells.

immune system (Kanno et al., 2012). Th cells can also be discriminated by the expression of characteristic transcription factors. The characteristic cytokines and transcription factors for each subtype of T helper (Th) cells are depicted in Figure 1.2.

Th1 and Th2 cells are the best described subtypes of Th cells. Th1 cells are specialized in the protection from viruses and other intracellular pathogens, whereas Th2 cells are crucial for the defense against the attack of extracellular pathogens such as parasites. In the last years, further subtypes, such as Th17, follicular B Helper T-cells (Tfh cells), and regulatory T-cells (Treg cells) have been described. Th17 and Tfh cells enhance the immune answer by secreting cytokines, such as IL-17 and IL-21, respectively (Fazilleau et al., 2009). In contrast, Treg cells attenuate the immune response to prevent uncontrolled immunity as well as the onset of autoimmune diseases (Vignali et al., 2008; Sakaguchi et al., 2010; Shevach, 2009). Because of their capability to prevent uncontrolled immunity, Treg cells are considered to be 'master

regulators' of immune homeostasis (Tang and Bluestone, 2008). Treg cells can develop within the thymus (natural Treg cells) or differentiate from naive Th cells (induced Treg cells). The latter type of Treg cells will be considered in this thesis. As the transcription factor forkhead factor P3 (FoxP3) is shaping the gene expression solely in Treg cells, it is used for discrimination between non-Treg cells and Treg cells (Bendfeldt, 2010).

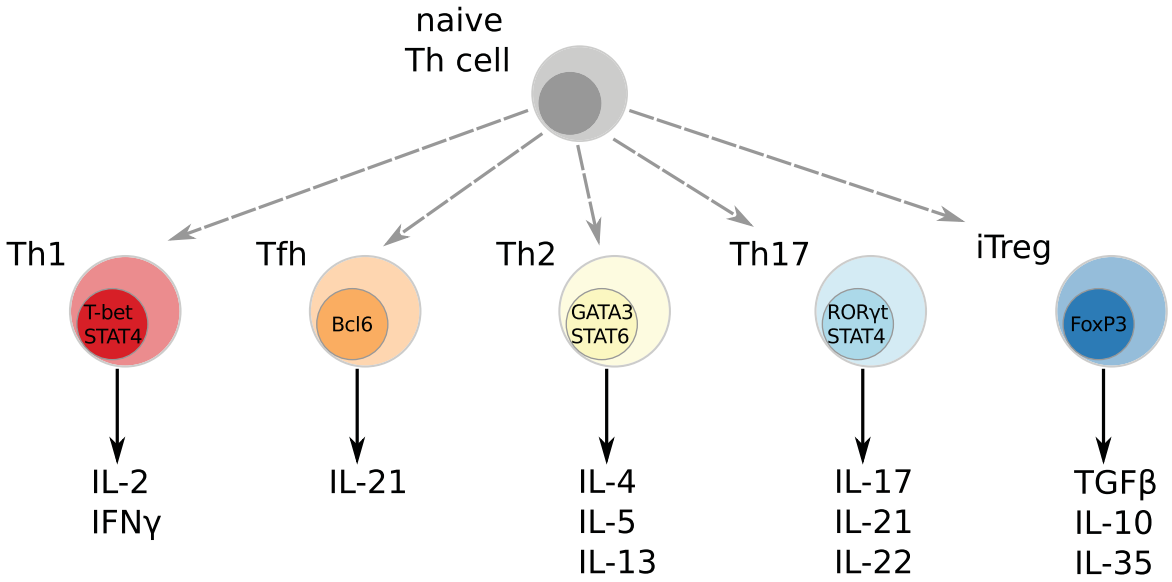

Figure 1.2.: Overview of the T helper cells. Simplified scheme of the differentiation of Th cells (gray dashed arrows). The five known Th subpopulations are depicted in color including their corresponding characteristic transcription factors and the secreted cytokines (black solid arrows).

1.2. Interleukin 2 balances immune responses

All of the described Th cells express a certain profile of cytokines, but only naive Th cells and Th1 cells are known to express the cytokine IL-2. Interleukin 2 shapes the magnitude and duration of immune responses. On the one hand, IL-2 activates immune responses, but on the other hand, IL-2 gene expression is part of negative feedback mechanisms attenuating the gene expression of IL-2 (Sojka et al., 2004; Villarino et al., 2007).

Protective immune responses activated by IL-2

In particular, IL-2 induces proliferation of B-cells, natural killer cells, and memory $CD4^+$ and $CD8^+$ T-cells by activating the transcription factors c-fos and c-myc (Gómez-Martín et al.,

2009; Grigorieva et al., 2000). Additional activation of anti-apoptotic proteins, such as the anti-apoptotic members of the Bcl-2 family, enhance the proliferative effect of IL-2 (Attema et al., 2002; Gaffen and Liu, 2004). IL-2 also enhances cytokine production and secretion as well as antibody secretion and stimulates the differentiation of various immune cell types, such as Treg cells and Th2 cells (Lan et al., 2008).

Peripheral immune tolerance mediated by IL-2

As IL-2 gene expression has profound effects on the activity of the immune system, constitutive IL-2 expression might lead to an uncontrolled immune answer. This uncontrolled immune answer might be fatal for the organism and thus needs to be prevented. To prevent constitutive IL-2 gene expression different mechanisms have evolved. For instance transient IL-2 response is established via paracrine signaling through the IL-2 receptor (Villarino et al., 2007). *In vivo* the secretion of IL-2 is almost completely down-regulated within 14 to 16 hours (Sojka et al., 2004). IL-2 also stimulates the pro-apoptotic Fas ligand pathway leading to activation-induced cell death (Gaffen and Liu, 2004; Villarino et al., 2007), which is defective in the absence of a functional IL-2/IL-2 receptor system (Schimpl et al., 2002). IL-2 is required for the development and function of induced Treg cells (Dooms and Abbas, 2010; Hoyer et al., 2008; Turka and Walsh, 2008). Furthermore, IL-2 inhibits the development of Th17 cells (Malek, 2008), which promote inflammatory reactions. By all these different mechanisms, constitutive IL-2 expression is prevented and peripheral tolerance is established.

IL-2 and its impact on disease

The diverse effects of IL-2 on protective immune responses as well as peripheral immune tolerance form the basis of its profound role in various diseases. The importance of IL-2 has been demonstrated in mice by showing that the administration of IL-2 reduces the severity of experimental autoimmune encephalomyelitis (EAE), which is an animal model of brain inflammation (Dooms and Abbas, 2010). In contrast, it seems to be difficult to induce EAE in IL-2 deficient mice (Schimpl et al., 2002).

IL-2 knock-out mice show a complex autoimmune phenotype including multi-organ inflammation (Chistiakov et al., 2008; Schimpl et al., 2002; Villarino et al., 2007). The lack of IL-2 is also associated with the human autoimmune disease systemic lupus erythematosus (SLE) (Crispín and Tsokos, 2009). Patients with SLE suffer from an autoimmune inflammation of almost any organ of the body (Wahren-Herlenius and Dörner, 2013). SLE is one case,

where the transcriptional regulation of IL-2 has been shown to be of physiopathogenic relevance (Gómez-Martín et al., 2009). The decreased levels of IL-2 prohibit the proliferation of Treg cells, as findings of several studies have identified reduced numbers and frequencies of Treg cells in SLE patients (Wahren-Herlenius and Dörner, 2013). Due to the reduced numbers of Treg cells, Th cells are hyperactivated and inflammation is induced (Schimpl et al., 2002).

Another example of the importance of IL-2 in disease is type I diabetes, which is studied in non-obese diabetic mice. At least two single nucleotide polymorphisms in intron 1 of the IL-2 gene are associated with type I diabetes (Chistiakov et al., 2008). IL-2 administration is used to prevent type 1 diabetes (Dooms and Abbas, 2010). IL-2 administration is also approved as clinical treatment in other diseases, such as skin melanomas and kidney cancer (Boyman et al., 2006).

On the other side, IL-2 needs to be suppressed by cyclosporin A (CsA) to prevent organ rejection after organ transplantation. These examples illustrate the complex involvement of IL-2 in disease due to its opposing roles in the regulation of immune responses.

1.3. Molecular mechanisms during the activation of T helper cells

Th cells are activated by APCs that present an antigen via the MHCII. The stimulation is forwarded into the Th cell via the TCR complex including CD4 and then conveyed into the nucleus via multiple signaling cascades. In the following section, I will explain in more detail the major signaling cascades, which are induced in Th cells by APCs. They lead to the activation of transcription factors that are responsible for the regulation of IL-2 gene expression.

Activation of the T-cell receptor and proximal T-cell receptor signaling

Antigen presenting cell (APC) use the major histocompatibility complex (MHC)II to present peptides to Th cells. The MHCII molecules form a complex with the T-cell receptor (TCR) and the CD4 molecule on the membrane of the Th cells. The cluster of differentiation 3 (CD3) molecules are associated with the TCR (hereafter referred to as TCR/CD3 receptor complex). Additional binding of agonists to the cluster of differentiation 28 (CD28) receptor, which is co-localized with the TCR/CD3 receptor complex (Acuto and Michel, 2003), increase the amplitude of the TCR/CD3 receptor complex-induced transcriptional response (Diehn et al., 2002). These molecules together with further regulatory molecules collectively form the immune synapse, where conformational changes of the receptor complexes transmit the

signal to a proximal intracellular signaling complex and activate a complex signaling network (Figure 1.3) (Rudolph et al., 2006). During proximal signaling, the immunoreceptor tyrosine based activation motifs (ITAMs) that are part of the CD3 molecules become phosphorylated by kinases, which are themselves activated by the TCR. In addition, the tyrosine kinase ζ-chain-associated protein kinase 70 (Zap70) phosphorylates adaptor proteins to support further complex formation at the inner side of the cell membrane. Within this complex the phospholipase C γ (PLCγ) is kept in a conformation of optimal activity (Smith-Garvin et al., 2009) to cleave the small signaling molecule phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerine (DAG) and inositol trisphosphate (IP_3). DAG is able to stimulate the rat sarcoma (Ras) pathway and the protein kinase C θ (PKCθ)-dependent pathway, whereas IP_3 activates the calcium-dependent pathway (Putney, 2012; Quintana et al., 2005) (Figure 1.3). Eventually, the signaling network activates the transcription factors nuclear factor of activated T-cells (NFAT), activator protein 1 (AP-1), and nuclear factor κ-light-chain enhancer of activated B-cells (NF-κB), which are crucial for initiating IL-2 gene transcription (Kim et al., 2006).

Calcium-dependent pathway

If IP_3 binds to its receptor at the endoplasmatic reticulum (ER), calcium is released from the ER (Figure 1.3, green pathway). Calcium triggers additional calcium influx via the calcium release-activated calcium channels (CRACs) leading to an elevated level of intracellular calcium (Quintana et al., 2005). Calcium binds to calmodulin and leads to the activation of the phosphatase calcineurin (Timmerman et al., 1996; Lewis, 2001). Calcineurin dephosphorylates the transcription factor NFAT, which plays a central role in regulating the gene expression of IL-2 (Rao et al., 1997). Recently it has been shown that calcineurin also dephosphorylates B-cell lymphoma/leukemia 10 (Bcl10), which links the NFAT activating pathway with the NF-κB activating PKCθ-dependent pathway (Frischbutter et al., 2011).

PKCθ-dependent signaling

PKCθ is recruited by the scaffolding protein caspase recruitment domain-containing MAGUK protein-1 (CARMA-1) into the immune synapse and becomes activated (Marsland and Kopf, 2008) (Figure 1.3, orange pathway). The active PKCθ triggers c-jun activation via a kinase cascade of Jun kinase kinase (JNKK) and subsequently c-Jun N-terminal kinase (JNK) activation. Furthermore, active PKCθ phosphorylates Carma, which then is able to recruit the complex consisting of Bcl10 and mucosa-associated lymphoid tissue lymphoma translocation

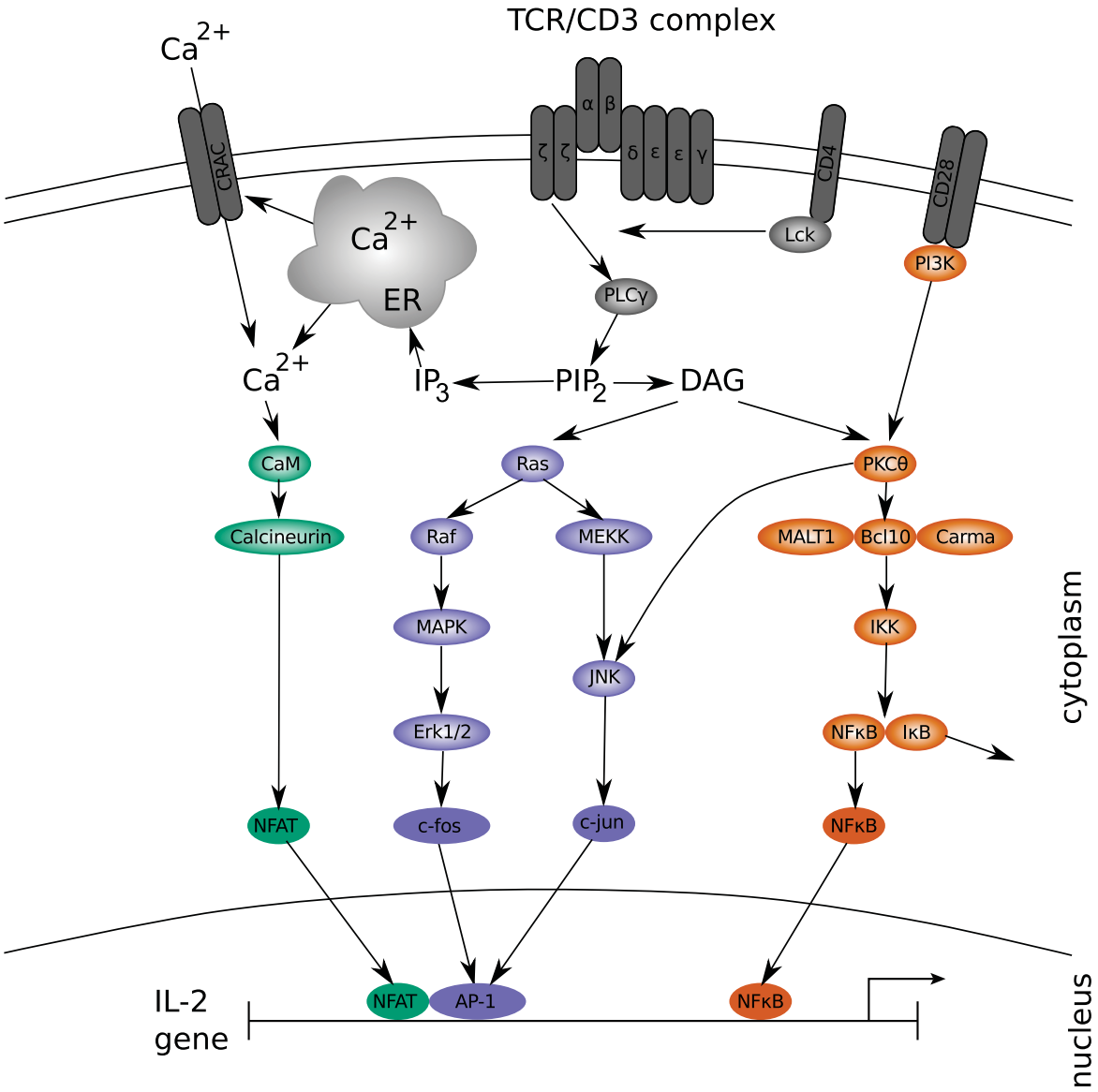

Figure 1.3.: Signal transduction in Th cells. Scheme of the three major signaling pathways resulting in induction of IL-2 gene expression: calcium-dependent pathway (green), Ras-dependent pathway (violet), and PKCθ-dependent pathway (blue). The receptor molecules and signaling molecules of the proximal pathway are depicted in gray (adapted from (Sieber and Baumgrass, 2009) and (Ghosh, 2011)). The signal transduction mechanisms are explained in detail in Section 1.3.

protein 1 (MALT1) to the membrane (Lobry et al., 2007; Thome et al., 2010). This complex activates IκB kinase (IKK). IKK phosphorylates nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) leading to its ubiquitination and degradation. When IκB is degradated, it stops inhibiting the transcription factor NF-κB and NF-κB can translocate into the nucleus (Altman et al., 2000; Weil and Israël, 2006). The activation pathway described above activates NF-κB heterodimers consisting of c-Rel and p65 subunits (Köntgen et al., 1995; Beinke and Ley, 2004).

Ras-dependent signaling

The small signaling molecule DAG leads to the activation of Ras-GTP-exchanging factor (RasGEF) (Figure 1.3, violet pathway). RasGEF in combination with the localization of Sos-growth factor receptor-bound protein (GRB) complex to the membrane induces an exchange of a guanosine diphosphate (GDP) with a guanosine triphosphate (GTP) at Ras and thus activates the GTPase Ras (Johnson and Chen, 2012). Ras activates kinases, such as the members of mitogen-activated extracellular-regulated kinase kinase (MEKK) family including Raf, which results in the induction of several phosphorylation cascades (Scheele et al., 2007). One cascade for instance activates the transcription factor extracellular-signal-regulated kinase (ERK). ERK in turn induces the gene expression of the transcription factor c-fos. Another cascade activates the JNK, which leads to the phosphorylation of the transcription factor c-jun. The two transcription factors c-fos and c-jun form the complex AP-1 (Jain et al., 1992a).

Mimicking the activation of Th cells *in vitro*

The activation of the TCR/CD3 receptor complex and its co-receptor CD28 can be simulated by the administration of Ionomycin and phorbol 12-myristate 13-acetate (PMA). Ionomycin causes a calcium influx leading to a saturated intracellular calcium level. The calcium influx activates calcium-dependent signaling pathways, such as the calcineurin pathway (Chatila et al., 1989). PMA activates PKCθ. The effects of PMA on PKCθ result from its similarity to DAG, which is one of the natural activators of PKCθ. In consequence of PMA and ionomycin administration, the Th cells become fully stimulated, although the proximal signaling complex is bypassed (Lin and Wang, 2004). In the experiments considered in this thesis, Th cells were either stimulated with CD3/CD28 or with PMA/Ionomycin.

In summary, CD3/CD28 stimulation or PMA/Ionomycin stimulation activate the transcription factors NFAT, AP-1, and NF-κB (via the complex signaling network shown in Figure 1.3),

which are central to the initiation of IL-2 gene transcription.

1.4. The transcriptional regulation of the Interleukin 2 gene

The expression of genes is regulated at different layers, such as the level of transcription, post-transcriptionally after the mRNA is formed, and on the level of translation (for a general review see (Sperling, 2007)). IL-2 is an inducible gene, i.e. it is expressed if an antigen is presented to the T-cell receptor. IL-2 has a predominantly transcriptionally regulated gene expression (Malek, 2008), which will be described in the following section.

Core promoter and distal enhancer of IL-2

The promoter region of IL-2 can be divided into two main parts. One is the distal enhancer region (Yui et al., 2001) and the second one is the proximal core promoter, which spans about 300 base pairs (bp) (Fuse et al., 1984; Brunvand et al., 1988). At the distal enhancer, transcription factors such as the NFκB subunit p65 can bind leading to a shift of a nucleosome, which covers the core promoter (Rao et al., 2003). If this nuclesome is shifted, then the core promoter is accessible for the binding of further transcription factors. The shift of the nucleosome takes place during the differentiation of naive Th cells to memory Th cells (Rao et al., 2003). At the core promoter, multiple activation-dependent response elements have been identified (Jain et al., 1992b; Ochi et al., 1994; Rothenberg and Ward, 1996; Serfling et al., 1989; Weaver et al., 2007), which are short cis-regulatory regions (Figure 1.4). The antigen receptor response element 1 & 2 (ARRE-1/2) includes transcription factor binding sites for NFAT (Figure 1.4, green) and AP-1 (Figure 1.4, violet), whereas the CD28 response element (CD28RE) includes binding sites for NFAT and NF-κB (Figure 1.4, orange) (Verweij et al., 1991). The core promoter harbors further binding sites for NFAT, AP-1, and NF-κB as well as for additional transcription factors, such as octamer binding transcription factor (Oct), and myocyte enhancer factor 2 (Mef). Although further transcription factors bind to the promoter region of IL-2, they are either available at all times in memory Th cells (e.g. Oct (Kamps et al., 1990; Murayama et al., 2006) or Mef (Pan et al., 2004; Liu, 2005)) or play a role in differentiation (e.g. acute myeloid leukemia (AML)) or anergy (e.g. Ikaros) (Crispín and Tsokos, 2009), rather than in activation of Th cells. I will focus on the most important transcription factors that play a role in the induction of IL-2 expression during Th cell activation, namely NFAT, c-fos, p-c-jun, and NF-κB, as well as the repressor FoxP3.

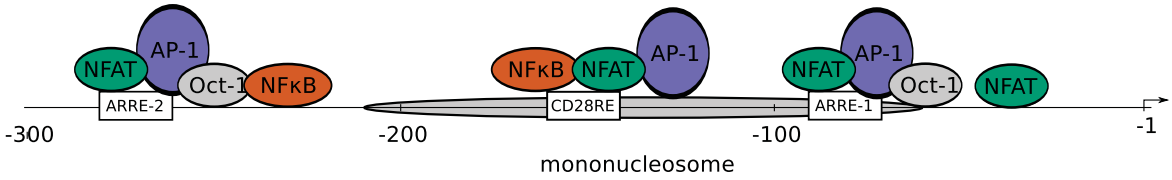

Figure 1.4.: Overview of the IL-2 core promoter. Shown is the proximal promoter from -300 bp to the transcription start site, including the response elements ARRE-1/2 and CD28RE. The binding sites for the activation-dependent transcription factors NFAT, AP-1, and NF-κB as well as for the activation-independent transcription factor Oct are shown. The position of a nucleosome covering the proximal promoter in naive Th cells is depicted in gray.

NFAT

The family of NFAT proteins consists of five family members, namely NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5 (Rao et al., 1997; Masuda et al., 1998; Macián et al., 2002). The first four are activated via calcium signals (thus considered in context of TCR signaling 1.3). In contrast, NFAT5 is activated by osmotic stress (Macián et al., 2002). Calcium binds calmodulin, which in turn activates the phosphatase calcineurin. In the case of NFATc2, this phosphatase is responsible for the dephosphorylation of 13 phosphorylation sites triggering a conformational switch of NFATc2 (Loh et al., 1996; Okamura et al., 2000). This conformational switch reveals a nuclear translocation site leading to the import of NFATs into the nucleus (Crabtree, 1999; Serfling et al., 2000). NFAT preferably binds deoxyribonucleic acid (DNA) in heterodimeric complexes, e.g. together with AP-1, Mef, or GATA binding transcription factor 4 (GATA4) (Macián, 2005). In the nucleus, NFAT is phosphorylated by several kinases, such as glycogen synthase kinase 3β (GSK3β) and protein kinase A (PKA), leading to its export and abrogation of the stimulus response (Beals et al., 1997; Crabtree and Olson, 2002; Serfling et al., 2006; Sumpter et al., 2008).

AP-1

The AP-1 transcription factor is a dimer, composed of Jun and Fos family proteins (Jain et al., 1992a; Shaulian, 2010). The Fos family consists of four proteins, namely c-fos, FosB, Fra-1, and Fra-2, whereas the Jun family consists of three proteins, namely c-jun, JunB, and junD (Jain et al., 1994). Usually, c-Jun and c-Fos form the complex AP-1 at the IL-2 promoter. AP-1 binds in association with other transcription factors, especially NFAT. The interaction between AP-1 and other factors enhances DNA binding affinity of each interacting partner (Kim et al.,

2006).

NF-κB

The NF-κB family consists of five different DNA binding subunits (Schmitz and Krappmann, 2006; Vallabhapurapu and Karin, 2009) having activating or inhibiting functions (Hailfinger et al., 2011). All members have a Rel homology domain (RHD), which includes amino acid sequences for dimerization, interaction with IκB, nuclear translocation as well as DNA binding (Schulze-Luehrmann and Ghosh, 2006). This DNA binding domain of the NF-κB family is similar to the DNA binding domain of the NFAT family (Serfling et al., 2004), but NF-κB binds to DNA with a higher binding affinity (Rooney et al., 1995; Serfling et al., 2000; Macián, 2005). Only the subunits p65, RelB, and c-Rel contain a c-terminal transactivation domain, whereas p50 and p52 lack the transactivation domains (Schulze-Luehrmann and Ghosh, 2006). In the following thesis, the subunit p65 will be considered as a representative for the NF-κB family.

FoxP3

The FoxP3 transcription factor is necessary and sufficient for the commitment of the Treg cell lineage (Chen et al., 2006). It is associated with the repression of cytokines, such as IL-2 and Interferon γ (IFNγ) as well as with the induction of receptors, such as the IL-2 receptor (CD25) and cytotoxic T-Lymphocyte antigen 4 (CTLA-4), which transmits an inhibitory signal to T-cells. (Chen et al., 2006; Marson et al., 2007). FoxP3 binds the DNA in a complex with NFAT leading to the repression of IL-2 gene expression (Wu et al., 2006; Hu et al., 2007; Shevach, 2009). In this thesis, the fluorescence intensity of FoxP3 has been used as a marker of Treg cells (Fontenot and Rudensky, 2005; Sakaguchi, 2005; Alberts et al., 2008).

Complex formation of transcription factors

The transcription factors AP-1, NFAT, and NF-κB do not necessarily bind to the DNA by themselves. NFAT for example, has been shown to bind in homo- and hetero-dimers using the isoforms NFATc1 and NFATc2. Furthermore, a crystallized structure of a NFAT and AP-1 complex exemplifies an additional way of complex formation. The transcriptional repressor FoxP3 is also able to form a complex with NFAT using the same binding site as AP-1 (Wu et al., 2006).

1.5. Detecting immune cells using flow cytometry

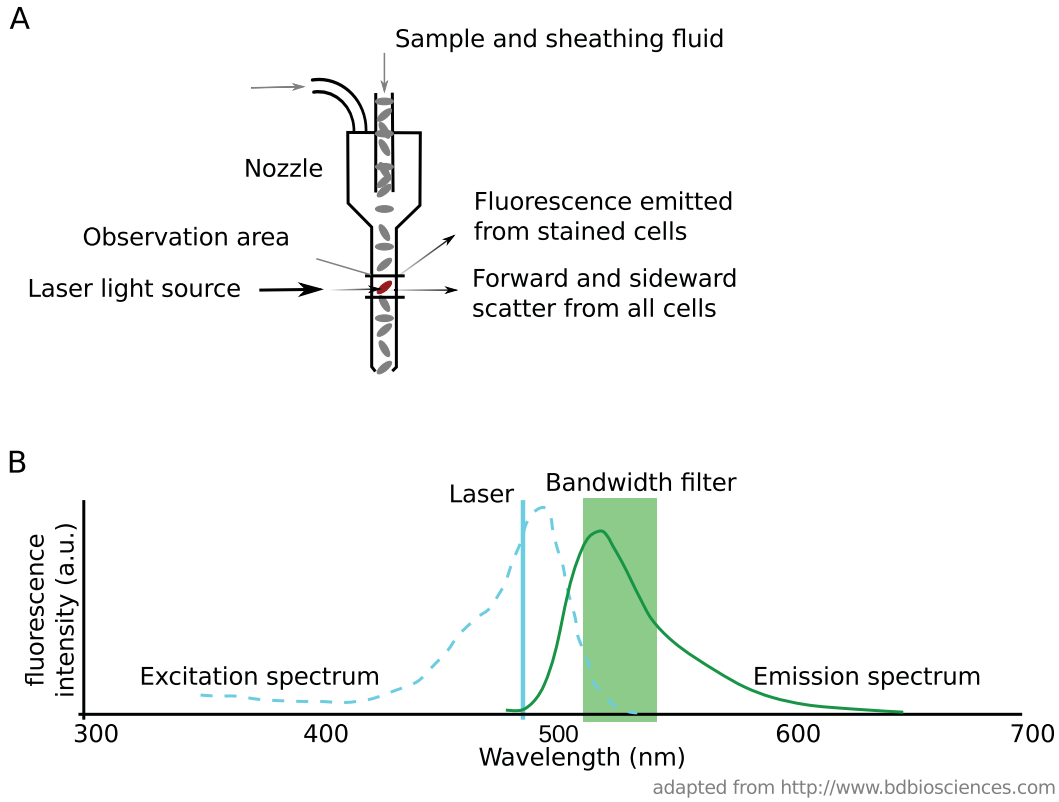

Figure 1.5.: Organization and function of a flow cytometer. (A) Cells are added together with a sheathing fluid to the flow cytometer nozzle, where cells are separated. Laser light of a single wavelength is directed at each cell. Depending on the single cell properties, scattered and fluorescent light can be detected in the observation area. (B) The excitation curve (blue dashed) and emission curve (green solid) of the FITC fluorophore is sketched. Also depicted is the wavelength of the applied laser and the bandwidth filter to detect the fluorescence intensity of the FITC fluorophore.

To sort cells according to the proteins expressed at their surface a flow cytometer is used (sketched in Figure 1.5A). In a flow cytometer, a sheathing fluid is added to the cells of a sample (BD-Biosciences, 2000). This sheathing fluid flows faster than the cells, which leads to the separation of the cells. In the observation area a laser light is directed at each passing cell. The laser light that is scattered by the cell provides information about the size of the cell (forward scatter) and the granularity, i.e. intracellular structure, of the cell (side scatter). Based on these properties, cells can be distinguished from debris as well as separated into different types of cells. In particular, different leukocyte subpopulations can be detected,

namely lymphocytes, monocytes, and neutrophils.

In addition, antibodies tagged with fluorophores can be used to detect levels of certain proteins expressed by a cell based on the emission of colored light (Figure 1.5B). The color of a laser excites specific fluorophores. The fluorophores than emit light at different wavelengths. With the use of a bandwidth filter a specific emission range of a fluorophore can be detected (Figure 1.5B) and thus proteins bound by an antibody coupled with this specific fluorophore can be detected. In this thesis, the following proteins were of major interest: IL-2, FoxP3, NFATc2, phospho(p)-c-jun, p65, and c-fos. Their labeling is described in Table 1.1. Due to technical limitations, IL-2 and FoxP3 could only be combined with one other transcription factor (NFATc2, p-c-jun, p65, and c-fos), as shown in the table. One further combination, which was used in Section 3.3.1 and in Section 3.3.2, is the co-staining between c-fos, NFATc2, and IL-2.

Note that fluorescence intensities can be interpreted as concentrations, assuming that the technical amplification from the residual light amplifier relates fluorescence intensities to concentrations on a linear scale. This is a crude approximation, as the relations between antibody staining, excitation and emission will introduce nonlinear relations between fluorescence intensities and concentrations. To address this problem, I have mainly considered transformed and normalized fluorescence data in my analyses as stated in Section 2.2.

1.6. Modeling approaches to study IL-2 gene expression

A major focus of modeling T-cell activation investigates early signaling events, beginning with the proximal events at the receptor, initiated by ligand binding. The first published model (Kaufman et al., 1999) started out with explaining both positive and negative signaling effects

Table 1.1.: Overview of measured proteins and used fluorophores, including their best excitation and emission wavelengths.

protein	fluorophore	abbreviation	excitation	emission
IL-2	Allophycocyanin	APC	650 nm	660 nm
FoxP3	Phycoerythrin	PE	488 nm	578 nm
c-fos	Fluorescein isothiocyanate	FITC	495 nm	528 nm
p-c-jun	Fluorescein isothiocyanate	FITC	495 nm	528 nm
NF-κB	Fluorescein isothiocyanate	FITC	495 nm	528 nm
NFATc2	Fluorescein isothiocyanate	FITC	495 nm	528 nm
	Phycoerythrin	PE	488 nm	578 nm

in T-lymphocytes using a logical modeling approach. The same approach has been used for the signaling network (Saez-Rodriguez et al., 2007). These networks are described using logical modeling and aim at describing the interactions, rather than the dynamics of the signaling components (Beyer et al., 2011). The dynamics of signal transduction have also been addressed in many different models using ordinary differential equations such as NFAT signaling (Salazar and Höfer, 2003), NF-κB signaling (Lipniacki et al., 2004) and MAPK signaling (Legewie et al., 2007). These models were not intended to focus specifically on T-cell action. The logical network of T-cell activation (Saez-Rodriguez et al., 2007) has been extended to include signaling through the IL-2 receptor (Beyer et al., 2011) and is regularly updated (Louis-Dit-Sully et al., 2012). These networks end at the level of activating transcription factors. The activation of the individual transcription factors has been studied in detail for NFAT and NF-κB (Fisher et al., 2006).

A logical modeling approach has also been used to construct a directed network model of genes regulating Th1 and Th2 cells (Pedicini et al., 2010) and investigate the attractors of the network as a way of describing T-cell differentiation.

Another focus consists in modeling cross-regulation between different T cell subsets via IL-2 secretion. The models include the dynamics of IL-2 molecules, which act as the growth factor that Th cells produce and use and Treg cells need to proliferate (Garcia-Martinez and Leon, 2010; Busse et al., 2010). In a similar scope, it has been investigated how the immune system can avoid T-cell-mediated autoimmunity without the risk of immunodeficiency (Butler et al., 2013). The main idea of this paper is that activated T-cells can detect IL-2 being secreted by other activated T-cells and begin to proliferate and respond only if a threshold number of T-cells are activated (Butler et al., 2013). In addition, a detailed model has been developed (Khailaie et al., 2013), which unifies several components developed in previous studies, such as IL-2 dependent proliferation of T-cells, programmed cell death, IL-2 competition between activated T cell and activated Treg cells, and Treg-mediated immune suppression. All these models assume a linear rate of IL-2 production within each cell.

In another paper (Kemp et al., 2007), a partial least squares regression (PLSR) analysis was used to understand the relationship between ligand avidity, downstream signaling dynamics, and IL-2 production. To do so the authors measured multiple proteins within the signaling cascades of T cell activation at multiple time-points and the secreted amount IL-2 after four hours of stimulation. Their analysis suggests that a linear PLSR model can describe the highly nonlinear TCR pathway, from the activation state of signaling molecules to an IL-2 output on

a population based approach (Kemp et al., 2007).

1.7. Research objectives

So far, modeling of IL-2 gene expression regulated by transcription factors in primary human T-cells has not been addressed. Within my thesis I will address this aspect and explore the regulation of IL-2 gene expression in more detail.

IL-2 has been suggested to have an all-or-none response. This has been shown for stimulation with PMA/Ionomycin measuring IL-2 after five hours of stimulation (Podtschaske et al., 2007). It is not clear how the IL-2 gene expression evolves over time after a stimulus has been encountered. Another aspect, which needs to be addressed is the question whether the experimental setup may affect the observed bimodality in IL-2 gene expression.

The transcription factors c-fos, NFATc2, p65, and p-c-jun are essential for IL-2 gene expression (Garrity et al., 1994; Hughes and Pober, 1996). Within a population these transcription factors are heterogeneously distributed. I will explore how transcription factor concentration affect the concentration of IL-2 within IL-2 producers. Presuming that similar general activation mechanisms of the IL-2 gene exist in each memory Th cell, the frequency of IL-2 producers within a population could be related to the probability of a cell to produce IL-2. Based on this assumption, I will investigate how the transcription factor concentration affects the frequency of IL-2 producing cells as a proxy for the probability of a cell to produce IL-2. Furthermore I will compare these effects of the transcription factors and I will try to identify differences in their regulatory impact and whether there is a difference between Th subpopulations.

It has also been shown, that the different transcription factors may form a diverse set of transcription factor complexes (Macián et al., 2001), which are able to activate as well as inhibit IL-2 gene expression. Thus I will furthermore explore how the interplay between the different transcription factors is related to IL-2 gene expression.

In order to approach these questions, I have collected experimental data on primary human Th cells using single cell flow cytometry and processed these data to perform my modeling workflow.

2. Methods

2.1. Purification and stimulation of memory T helper cells and data acquisition

In this study, data of primary human memory T helper cells (memory Th cells) from multiple donors were collected. They express the transmembrane protein CD4, which is used as a marker to detect Th cells in a population of lymphocytes. To discriminate memory Th cells from naive Th cells, isoforms of the transmembrane phosphatase CD45 are used: while the latter express the CD45RA variant, memory Th cells present the CD45RO isoform at their surface. For their purification from human blood samples, a depletion assay with a mixture of antibodies coated to magnetic beads are used in a cell sorting procedure. The antibodies recognize surface proteins specifically leading to a memory Th cell purity of 98% (Milteniy Biotech).

To induce IL-2 gene expression as explained in Section 1.3, memory Th cells were either stimulated with CD3/CD28 or with PMA/Ionomycin, mimicking T-cell activation signals (Section 1.3). The receptor-transmitted stimulation has been conducted using magnetic beads, which were coated with anti-CD3 and anti-CD28 antibodies (25 μl for 10^6 cells). Otherwise 10 $\frac{\text{ng}}{\text{ml}}$ of PMA and 1 $\frac{\mu g}{\text{ml}}$ Ionomycin have been used. Cells treated with dimethyl sulfoxide (DMSO) were used as unstimulated control sample. To measure the concentration of the cytokine IL-2 produced by one cell, the secretion of IL-2 out of the cell was blocked using 5 $\frac{\mu g}{\text{ml}}$ Brefeldin A. Brefeldin A was administered at the time of stimulation, except for one experimental setup (Section 3.1), in which Brefeldin A was added 30 min before harvesting the cells.

After a defined time of stimulation (Table 2.1), the memory Th cells were harvested, fixed, permeabilized, and stained with fluorophore-coupled antibodies (Table 1.1) as described in the doctoral thesis of Dr. Hanna Bendfeldt (Bendfeldt, 2010). Following this protocol, I conducted the time-series experiments in the group of Prof. Dr. Ria Baumgrass at the German

Rheumatism Research Centre (DRFZ) in Berlin, Germany. Furthermore, Dr. Hanna Bendfeldt and Dr. Tobias Scheel kindly provided additional data of memory Th cells stimulated with PMA/Ionomycin for 5 hours.

Flow cytometric measurements were performed using fluorescence-activated cell sorting (FACS)-Calibur flow cytometer (further details on flow cytometry are provided in Section 1.5). Flow cytometry data were analyzed with FlowJo® software and the statistics software R (Section 2.2).

2.2. Data preparation and normalization

The memory Th cells were gated for live cells and 0.5% of the cells with lowest or highest fluorescence intensities were removed. Raw fluorescence intensities were transformed using the asinh-function (which is the inverse of the hyperbolic sine, Equation 2.1), as suggested by Trotter (Trotter, 2007) and by Herzenberg *et al.* (Herzenberg et al., 2006)

$$\operatorname{asinh}(x) = \log(x + \sqrt{x^2 + 1}) \tag{2.1}$$

This transformation of the raw fluorescence intensity x has linear behavior around zero and log-like behavior in higher ranges of x and is more applicable than the widely used lognormal transformation (Limpert et al., 2001). The asinh transformation has been used with microarray data (Huber et al., 2002) and has also been proposed for flow cytometry data because of its capability to stabilize variances of data sets (Trotter, 2007).

The memory Th cell population was separated into memory effector Th cells (Teff cells) and regulatory T-cells (Treg cells) based on the fluorescence intensity of FoxP3 (Figure 2.1). In particular, a binary threshold has been applied manually to a data set of memory Th cells, which where harvested 5 hours after stimulation. This threshold was than applied to all other data sets and visually checked for consistency. I have used a similar threshold approach for the

Table 2.1.: Overview of the stimulation time, after which the cells were harvested for data acquisition. Unstimulated cells were either harvested after 5 hours (as control in the PMA/Ionomycin stimulation) or after 24 hours (as control in the CD3/CD28 stimulation).

stimulation	**stimulation time**							
PMA/Ionomycin	0 h	30 min	1 h	2 h	3 h	4 h	5 h	6 h
CD3/CD28	0 h	2 h	4 h	6 h	8 h	13 h	24 h	

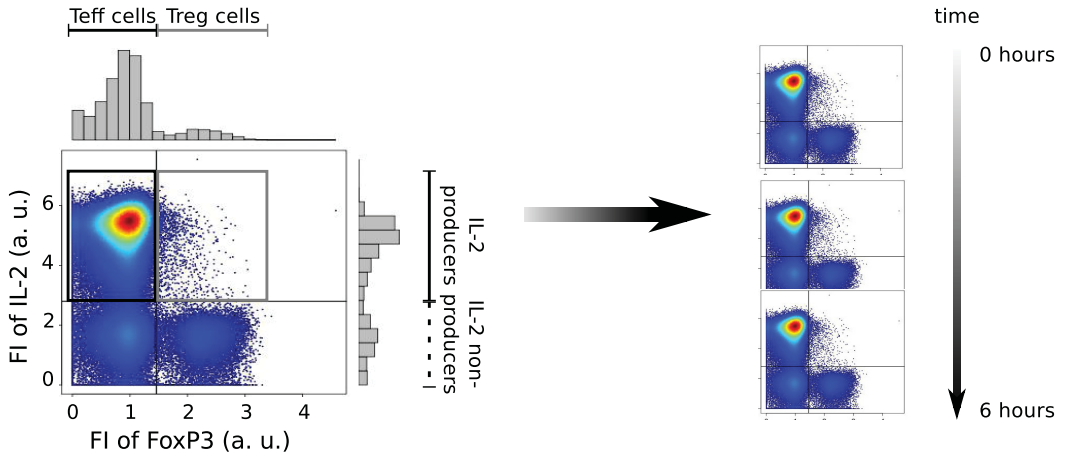

Figure 2.1.: Gating strategy for memory effector Thelper cells and regulatory memory Thelper cells and for IL-2 producing cells. Example scatterplot showing the fluorescence intensity (FI) of FoxP3 and IL-2 and the gating strategy for memory effector Th (Teff) vs. regulatory T-cells (Treg) subpopulation and the gating for IL-2 producing cells. The gating strategy was applied to all data sets in this thesis.

separation of IL-2 producing cells and IL-2 non-producing cells.

The transformed raw fluorescence intensities were mean shifted by the mean of the unstimulated data set to compare data from different donors and from different time points. The normalized data were used for the correlation analysis (Section 3.2.1) as well as further processed by quantile partitioning and used for a ranking of transcription factors (Section 2.6 and 3.2.5).

As the fluorescence intensities vary from donor to donor (Section 3.2.1) the data from the donors need to be transformed to allow a pooling for the modeling part (Section 3.2.3, 3.2.4). For the Teff cells as well as the Treg cells, the fluorescence intensities of each staining were z-normalized which allows pooling data from different donors. Note that in this case, data from different time-points and from the two subpopulations enclosing Teff cells and Treg cells are not directly comparable anymore.

2.3. Partitioning populations

To investigate the effect of transcription factor concentrations on the number of IL-2 expressing cells, each subpopulation of memory Th cells was partitioned into multiple bins according to the fluorescence intensity of each measured transcription factor.

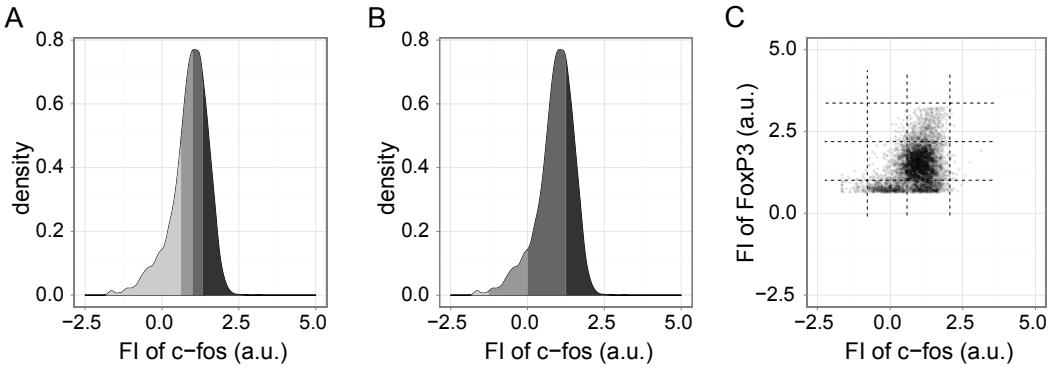

Figure 2.2.: Different approaches to partition cell populations based on fluorescence intensity. (A) The population of cells is partitioned based on quantiles of the fluorescence intensity (FI) of the transcription factor c-fos. (B) The fluorescence intensity of the transcription factor c-fos is divided into ranges of equal width. (C) The cell population is partitioned based on the fluorescence intensities of the transcription factors c-fos and FoxP3 using an equidistant grid (black dashed lines). The fluorescence intensities of c-fos and FoxP3 are used as an example.

For a qualitative analysis (Section 3.2.2), the partitioning was based on quantiles of fluorescence intensities (Figure 2.2A), meaning that each bin contains the same amount of cells. This allows the generation and comparison of histograms of IL-2 fluorescence intensities.

In a subsequent step, flow cytometric data were used for a semi-quantitative analysis (Section 3.2.3 and Section 3.2.4). In that case, the range of fluorescence intensities was partitioned into bins of equal width (Figure 2.2B). The equidistant partitioning has also been used for the fluorescence intensities of two transcription factors simultaneously (Figure 2.2C). The resulting data have been employed in the analysis of cooperative regulation of IL-2 gene expression (Section 3.3.1) as well as the inhibiting regulation of IL-2 gene expression by FoxP3 (Section 3.3.3). The equidistant partitioning of a population of cells might lead to bins containing no or very few cells. The frequency of IL-2 producing cells was only calculated for bins, which contained at least three cells.

2.4. Correlation analysis

To detect a correlation between the fluorescence intensity of the measured transcription factors and frequency of IL-2 producers, several correlation coefficients are applicable. The most

commonly applied one is Pearson's correlation ρ coefficient:

$$\rho_{\text{TF,IL-2}} = \frac{cov(\text{TF}, \text{IL-2})}{\sigma_{\text{TF}}\sigma_{\text{IL-2}}} \tag{2.2}$$

In order to use Pearson's correlation coefficient, one assumes that the data follow a Gaussian distribution and that there exists a linear correlation, which one wants to detect. In contrast, Spearman's rank correlation coefficient r only assumes a monotonic function between two variables X and Y. The values of the two variables are transformed into rank values, denoted by small letters x and y.

$$r = \frac{\sum_i (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_i (x_i - \bar{x})^2 \sum_i (y_i - \bar{y})^2}}, \tag{2.3}$$

where x_i and y_i are the individual rank values and $\bar{x}$ and $\bar{y}$ their respective mean.

If Spearman's correlation coefficient is greater in absolute values than Pearson's correlation coefficient it can suggest a nonlinear correlation.

2.5. Linear regression

An extension to a correlation analysis is linear regression, which is a simple model that explains the behavior of a dependent variable $\hat{y}$ using a linear combination of the predictors x_i

$$\hat{y} = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \epsilon_i, \tag{2.4}$$

In this particular case, the frequency of IL-2 producers is the dependent variable, which should be explained by the fluorescence intensities of regulating transcription factors. Hence, the fluorescence intensity of the measured transcription factors are the predictors. The goodness of a linear regression is defined by the explained variance R^2

$$R^2 = \frac{\sum_{i=1}^{n} (\hat{y} - \bar{y})^2}{\sum_{i=1}^{n} (y - \bar{y})^2}, \tag{2.5}$$

with y being the data points; in particular, y are the frequencies of IL-2 producers. $\bar{y}$ is in this case the mean frequency of IL-2 producers. And $\hat{y}$ are the estimated frequencies of IL-2 producers based on the linear regression model including the fluorescence intensities of the measured transcription factors. If the predictors are uncorrelated, Pearson's correlation

coefficients $\varrho(x_i, y)$ are proportional to the explained variance of the linear regression model.

$$R^2 = \sum_{i=1}^{n} \varrho(x_i, y)^2 \tag{2.6}$$

The explained variance can be decomposed, such that each predictor is corresponding to a partition of the explained variance.

2.6. Dispersion measure for ranking

The dispersion measure (D), also described as relative importance, decomposes the R^2 of the full model into contributions from the different regressors of the model (Groemping, 2007). This partition of the explained variance R^2 can be seen as the impact of a predictor x_i on a dependent variable $\hat{y}$. Based on the given data sets (Section 2.1), this linear regression approach is used in this thesis to dissect the impact of each transcription factor on the frequency of IL-2 producers (Section 3.2.5). The dispersion measure for a given predictor is the average over the average contributions of a predictor in linear regression models with different number of predictors (Equation 2.7) Groemping (2006). Therefore, the dispersion measure describes not only the direct effect of a regressor but also indirect effects of that regressor adding to a smaller model.

$$D(x_k) = \frac{1}{p} \sum_{j=0}^{p-1} \left(\sum_{\substack{S \subseteq \{x_1,\ldots,x_p\} \setminus \{x_k\} \\ n(S)=j}} \frac{seqR^2(\{x_k\} \mid S)}{\binom{p-1}{j}} \right), \tag{2.7}$$

where p is the number of possible models using the available regressors and $seqR^2$ is the sequentially added explained variance when adding the regressors with indices in M to a model that already contains the regressors with indices in S

$$seqR^2(M|S) = R^2(S \vee M) - R^2(S). \tag{2.8}$$

The explained variance R^2 in this context is a set of functions, which describes multiple linear models and each individual R^2 is defined by the set of regressors used in the linear model. Using this definition of the dispersion measure, the explained variance R^2 can be divided into the individual contributions of each predictor. In the performed analysis (Section 3.2.5), the considered data sets contain co-stainings of two transcription factors (i.e. two predictors) and

IL-2 (i.e. the dependent variable). In the case of two predictors x_1 and x_2, the dispersion measure simplifies to

$$D(x_1) = seqR^2(\{x_1\} \mid \{\}) + seqR^2(\{x_1\} \mid \{x_2\}) \tag{2.9}$$

which is equal to

$$D(x_1) = R^2(x_1 \vee x_2) - R^2(x_2). \tag{2.10}$$

Equation 2.10 implies, that the dispersion measure of one predictor equals the explained variance of both variables minus the correlation coefficient of the second predictor. This approach can be extended to the generalized explained variance in a similar fashion, but is not considered in this thesis. The R package `relaimpo` is used for the calculation of the dispersion measure. This package offers multiple variants of the dispersion measure, and I have used the variant based on the original definition of the dispersion measure (Lindeman et al., 1980).

2.7. Modeling the probability of inducing the IL-2 gene expression

An often used assumption is, that the rate of transcription is a function of the concentrations of the regulating transcription factors. In the case of IL-2, however, I assume that the rate of gene expression remains constant after initiation of transcription (Smith, 2004; Podtschaske et al., 2007). Based on the analysis in Section 3.1, I assume that transcription factors regulate only whether IL-2 is expressed and not how much. The probability that one cell expresses IL-2 can be related to the number of cells expressing IL-2 within a population. Consequently, I model the frequency of IL-2 producers, which express IL-2 above a certain threshold instead of IL-2 expression level as a function of transcription factor concentrations.

In this section, I will introduce the models, which describe possible mechanisms for the initiation of the IL-2 gene expression. IL-2 gene expression may be initiated by three types of mechanisms, which are either independent of a certain TF (type 1) or depend on TF binding to one (type 2) or multiple (type 3) binding sites (Figure 2.3). In addition, several heterodimeric transcription factor complexes have been reported to be involved in the regulation of IL-2 gene activation (Section 1.4). To model the concerted action of two transcription factor species coactivation models and competitive inhibition models are considered (Figure 2.4).

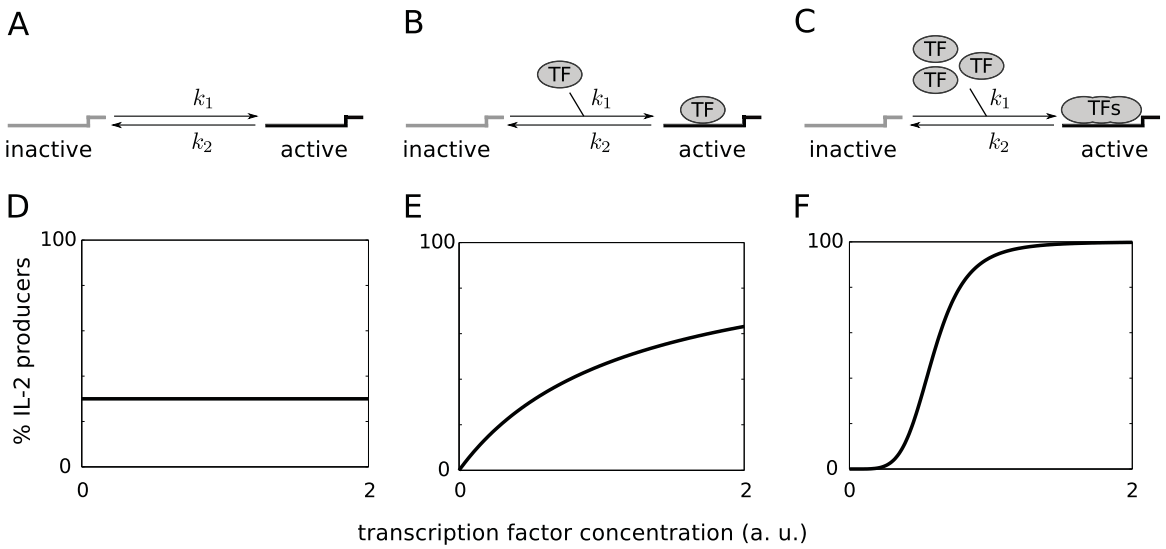

Figure 2.3.: Modeling approaches for IL-2 gene activation. The IL-2 gene switches between an inactive and an active state. (A-C) The schemes show how different activation mechanisms could work: (A) independent activation, (B) activation by one transcription factor at one binding site, and (C) cooperative activation by one transcription factor on multiple sites in a cooperative manner. In the lower panel is shown, how the percentage of IL-2 producers is predicted in dependency of the model with (D) independent activation, (E) activation by one transcription factor, and (F) cooperative activation.

Single transcription factor models:

(1) Independent model

In general the assumption is, that a gene switches between two states: a state in which transcription does not take place (inactive state) and a state in which the polymerase binds to the transcription start site and transcription is possible (active state). If the switching does not depend on the binding of any transcription factor, than the probability of the gene to be in either state can be modeled using the activation rate k_1 and the inactivation rate k_2 (Figure 2.3A). Assuming further that the activation of the IL-2 gene is fast (that is, claiming the steady-state of the active state), the probability of IL-2 gene expression simplifies to

$$\hat{y} = \frac{k_1}{k_1 + k_2} = b, \tag{2.11}$$

where $\hat{y}$ is the probability of the IL-2 gene to be in the active state, which corresponds to the percentage of IL-2 producers on a population scale. The parameters k_1 and k_2 are the activation rate and inactivation rate, respectively (see Appendix A for derivation of Equation 2.11).

(2) Michaelis-Menten model

If one transcription factor binds to the DNA and thus facilitates the activation of the gene (Figure 2.3B), I use a mass-action kinetic to describe this process. It is reasonable to assume a saturation of the transcription factor as it can be modeled with a Michaelis-Menten-like equation (Figure 2.3E, Appendix A)

$$\hat{y} = \frac{a \cdot x}{c + x} + b, \tag{2.12}$$

where b is a basal switching constant similar to that of the independent model. The parameter $a + b$ is the maximum probability for the IL-2 gene to be in the active state. The parameter c is similar to the Michaelis-Menten constant describing the concentration of the transcription factor x, at which equation 2.12 is at half between the basal frequency and the maximal frequency. A detailed explanation of the derivation of equation 2.12 is presented in Appendix A.

(3) Hill-like model

If multiple transcription factor binding sites are available, the steady-state of the IL-2 gene to be in an active state can be described by a Hill-like model (Figure 2.3C and Appendix A). The original model equation was first used to describe the binding of oxygen in erythrocytes (Hill, 1913), but has also be used for other systems where a similar mechanism was plausible (Salazar and Höfer, 2003; Chu et al., 2009; Mirny, 2010). The Hill-like equation

$$\hat{y} = \frac{a \cdot x^n}{c + x^n} + b \tag{2.13}$$

is similar to the Michaelis-Menten-like equation (Equation 2.12) with b being the basal switching constant, c being the Michaelis-Menten constant, and $a + b$ being the maximum probability to be in the active state. In addition, the parameter n influences the steepness of the curve at its point of inflection and is used as a measure of cooperativity in the system.

Transcription factor complex models:

(1) Activation models

To model possible interactions of the transcription factors c-fos and NFATc2, I assume one region for NFAT binding and one region for c-fos binding. Thus I can distinguish between four possible states:

1. neither NFAT nor c-fos is bound to the DNA,
2. only NFAT is bound to the DNA,
3. only c-fos is bound to the DNA, and
4. both transcription factors are bound to the DNA.

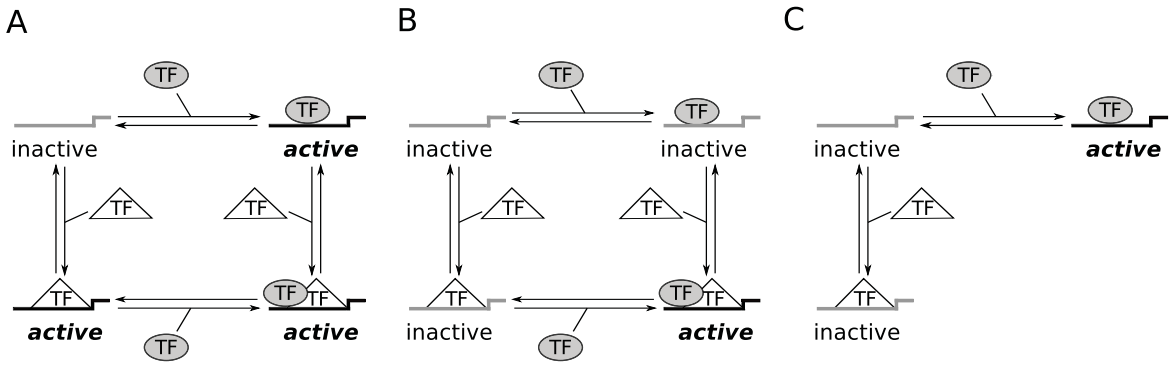

Figure 2.4.: Modeling approaches for mutual regulation of IL-2 gene activation. The IL-2 gene switches between an inactive and an active state. Whether the gene switches into an active state (black) depends on the binding of two different transcription factors. (A-C) The schemes show how different activation mechanism could work: (A) independent activation of the two transcription factors, (B) cooperative activation by two different transcription factors, and (C) competitive inhibition of one transcription factor (gray circle) by another transcription factor (white triangle).

If only one of the two transcription factors needs to be bound to the promoter, than three of the four possible states of the IL-2 gene would contribute to an activation of the IL-2 gene (Figure 2.4A). In a boolean modeling approach, such an independent activation model would be described by an OR-Gate, i.e. NFAT or c-fos are needed for IL-2 gene activation. Considering a second scenario, in which NFAT and c-fos only bind together at the promoter region of IL-2 and activate IL-2 gene expression, only state four would contribute to the gene expression of IL-2 (Figure 2.4B). The corresponding boolean description would use an AND-Gate in this case.

The OR-Gate and AND-Gate can be described by generic arithmetic functions and thus I used a multiplicative (Equation 2.14) and an additive (Equation 2.15) approach to describe the independent activation mechanism (Figure 2.4A) and the cooperative activation mechanism (Figure 2.4B), respectively.

$$\hat{y} = a_1 \cdot f_1(x_1) \cdot f_2(x_2) + b_{adj} \tag{2.14}$$

$$\hat{y} = a_1 \cdot f_1(x_1) + a_2 \cdot f_2(x_2) + b_{adj} \tag{2.15}$$

I relax the boolean approach as the concentrations of each transcription factor might have intermediate values between zero and one. Also, each transcription factor x_1 and x_2 by itself might have a different mechanism of regulating the activation of the IL-2 gene, which are described by the functions $f_1(x_1)$ and $f_2(x_2)$. Potential realizations of $f_1(x_1)$ and $f_2(x_2)$ may be given by Equation 2.11-2.13, depending on the assumed underlying mechanism of regulation. The combination of two different mechanisms might result in a basal switching rate not reflecting the actual switching rate. The heuristic models described in Equation 2.14-2.15 thus have one adjusting parameter for the basal switching rate b_adj.

(2) Competitive inhibition model

To describe the competitive inhibition of FoxP3, I use the fact that FoxP3 is able to bind at the DNA together with NFAT (Wu et al., 2006) and thus repressing IL-2 gene expression. This mechanism resembles a competitive inhibition model similar to enzyme kinetics with one variable x_1, which resembles an activating transcription factor, such as c-fos, NFATc2, NF-κB, or p-c-jun. The other variable x_2 resembles the transcription factor FoxP3, which inhibits the activation of the IL-2 gene (Figure 2.4C).

$$\hat{y} = \frac{a \cdot x_1 + b}{1 + c_1 \cdot x_1 + c_2 \cdot x_2} \tag{2.16}$$

where b and $\frac{a}{c_1}$ correspond to the basal and the maximal response level, respectively. The parameters c_1 and c_2 reflect the influence of the activating and the inhibiting transcription factors. The variable x_1 represents the concentrations of the activating transcription factors c-fos, NFATc2, NF-κB and the variable x_2 corresponds to the concentration of FoxP3. The dependent variable $\hat{y}$ reflects the fraction of cells, which are able to express IL-2 and therefore should be in the range between 0 to 1.

2.8. Model selection

As the different models explained in Section 2.7 suggest different mechanisms for the regulation of the probability that IL-2 will be expressed, it is necessary to distinguish how well each model explains the data.

The three models (Equation 2.11 - 2.13), in which the probability of IL-2 gene expression is dependent only on one transcription factor are nested models. The Michaelis-Menten-like (MM) model is more general than the independent model and the Hill-like model is more general than the MM model. The more general model with more parameters will always be able to fit the data at least as well as the model with fewer parameters. Thus, typically the Hill-like model will give a better fit to the data (i.e. lower residual sum of squares) than the MM model, which in turn will give a better fit than the linear model. One approach to determine, which model is significantly better, is using an F-test. If there are n data points to estimate parameters of two models from, then one can calculate the F-statistic, given by

$$F = \frac{\left(\frac{\mathrm{RSS}_1 - \mathrm{RSS}_2}{p_2 - p_1}\right)}{\left(\frac{\mathrm{RSS}_2}{n - p_2}\right)}, \tag{2.17}$$

where p_1 and p_2 are the number of parameters in model 1 and model 2 with model 2 being the more general one. RSS_1 and RSS_2 are the residual sums of squares of the two nested models. The residual sum of squares is defined as

$$RSS = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2, \tag{2.18}$$

where y_i are the measured data and $\hat{y}_i$ are the estimated data. If the regression model has been calculated with weights, then the RSS is replaced with χ^2, the weighted sum of squared residuals. Under the null hypothesis that the more general model does not provide a significantly better fit than the more restricted model, F will have an F-distribution, with (p2-p1, n-p2) degrees of freedom. This null hypothesis is rejected if the F calculated from the data is greater than the critical value of the F-distribution for some desired false rejection probability, e.g. 0.05 as assumed in this thesis.

In case of the models with two transcription factors it is not possible to use the F-test as the models for cooperative activation (Equation 2.15 and 2.14) are not nested. To compare the models I use Akaike's information criterion, corrected for sample size (AICc) (Equation 2.19), which takes into account the distance between model and data and considers the complexity of

the model (Burnham and Anderson, 2002). With increasing complexity of a model (increasing numbers of parameters) it is easier to explain the data but more difficult to predict data (an effect called overfitting).

$$AICc = n \cdot \ln(\mathcal{L}) + 2k + 2k\frac{k+1}{n-k-1}, \tag{2.19}$$

where, n represents the number of data points and k represents the number of parameters in the given model. $\mathcal{L}$ is the likelihood of the model representing the data. In a fitting procedure the likelihood can be estimated by the residual sum of squares (RSS) if the errors (σ_i) at each data point are assumed to be identical. In contrast to the F-test, the AICc is not restricted to nested models (Burnham and Anderson, 2002).

For calculation the nonlinear fitting procedure 'Isqnonlin' from Matlab was used. Starting parameters were randomly sampled from a standard normal distribution and the fit with the smallest AICc was used.

3. Results

3.1. Measuring and analyzing time-series data of Interleukin 2 gene expression

IL-2 is expressed in an all-or-none fashion, which was shown by flow cytometry (Podtschaske et al., 2007). In these flow cytometry experiments, the all-or-none expression of IL-2 has been observed at only one time point (5 hours) after stimulation with PMA and Ionomycin (PMA/Ionomycin). In order to better understand the dynamics of IL-2 gene expression that result in the all-or-none pattern, I investigate the dynamics of IL-2 gene expression upon stimulation with PMA/Ionomycin as well as with CD3/CD28 in memory T helper cells (Section 1.1). I focus on memory effector T helper cells (Teff cells) and regulatory T-cells (Treg cells) in the memory Th cell population, and the fluorescence intensity of FoxP3 is used to distinguish between these two subpopulations (Section 2.2, Figure 2.1). FoxP3-expressing cells indicate Treg cells; the remaining cells correspond to the Teff cells. In the analysis of Teff and Treg subpopulations, I focus on the number of cells, which express IL-2 above a certain threshold (hereafter referred to as IL-2 producers).

In both Teff cells and Treg cells first IL-2 producers are detectable after approximately one hour of stimulation with PMA/Ionomycin (Figure 3.1A). The number of IL-2 producers increases monotonically, both in Teff cells and Treg cells, until it reaches a plateau at four hours after stimulation. Within the six hours of measurements, no decrease in the number of IL-2 producers can be detected. However, the level of the plateau differs between the two cell types: While on average about 70% of Teff cells produce IL-2, the frequency of IL-2 producers lies about 15% in Treg cells (Figure 3.1A). In contrast to this, when stimulating memory Th cells with CD3/CD28, IL-2 producers are detectable within two hours of stimulation in both subpopulations (Figure 3.1B). The number of IL-2 producers saturate after eight hours of stimulation in Teff cells, but increase monotonically in Treg cells.

In these experiments (Figure 3.1A and 3.1B), the secretion of IL-2 was blocked by Brefeldin A

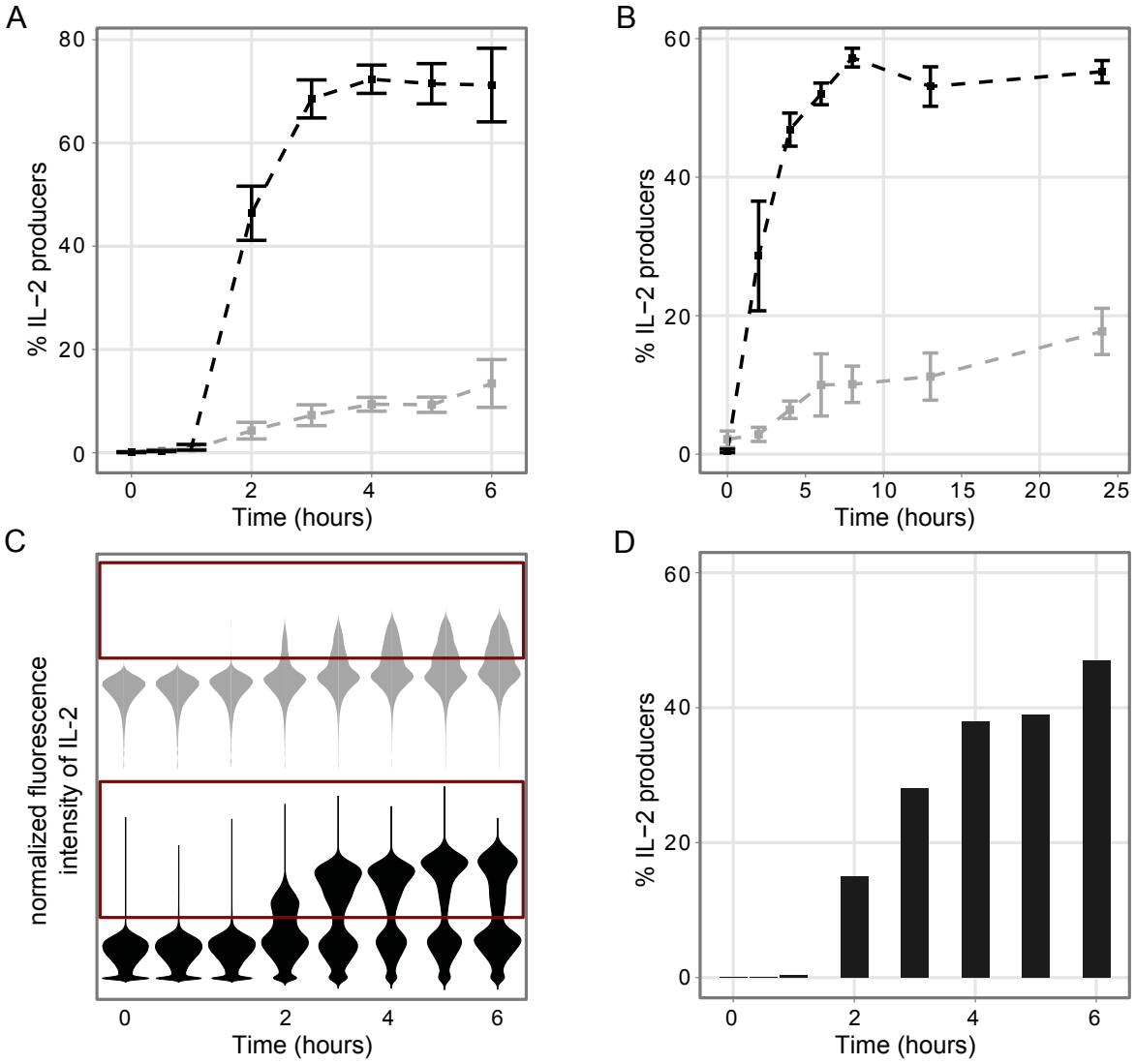

Figure 3.1.: Time course of activated memory T helper cells. (A) The fraction of IL-2 producing cells (in percent) after PMA/Ionomycin stimulation in Teff cells (black) and Treg cells (gray) is shown. Cells were stimulated for up to six hours. The bars denote the standard deviation from five replicates. The black and gray lines are for guidance of the eye. (B) The fraction of IL-2 producing cells after CD3/CD28 stimulation is shown. Cells were stimulated for up to 24 hours. The bars denote the standard deviation from five replicates. The black and gray lines are for guidance of the eye. (C) Double-sided density plot for fluorescence intensity (FI) of IL-2 over time with long (black) and short (gray) Brefeldin A admission. The red rectangles denote the IL-2 producers. (D) The fraction of IL-2 producing cells after PMA/Ionomycin stimulation in memory T helper cells with short Brefeldin A admission (n=1).

for the whole time of stimulation and thus the secretion can be neglected in the time of stimulation. In consequence, the concentration of IL-2 measured in one cell can be assumed to depend only on the production rate and degradation rate of IL-2. The degradation rate of IL-2 is $0.17\frac{1}{h}$ as the half-life is roughly 4 hours (Bill et al., 1994) after PMA/Ionomycin treatment. That is within the time frame of the time-course measurements. Therefore, if Brefeldin A is applied to inhibit IL-2 secretion, all cells, which at one point express IL-2, are at the time point of measurement counted as an IL-2 producing cell even if IL-2 production has ceased already. Thus, the bimodality of IL-2 could be due to a cumulative effect imposed by Brefeldin A. In order to avoid this potential cumulative effect, IL-2 secretion was blocked only in the final 30 minutes of cell culturing (referred to as short Brefeldin A admission) in a subsequent experiment.

In Figure 3.1C, the density of IL-2 fluorescence intensity is shown over time as double-sided density plot. However, the fluorescence intensity of IL-2 shows bimodal behavior with both long (black) and short (gray) Brefeldin A admission. The bimodal behavior of IL-2 despite shorter Brefeldin A admission demonstrates that the cumulative effect of blocking IL-2 secretion might be excluded as a reason for bimodality in IL-2 gene expression. Rather, Brefeldin A admission affects the number of IL-2 producers (see Figure 3.1D). While short admission results in roughly 45% IL-2 producers in memory Th cells after six hours of PMA/Ionomycin stimulation, their fraction is larger with full Brefeldin A admission ($63.2\% \pm 7.6\%$).

3.2. Dissecting impact of individual transcription factors on Interleukin 2 regulation

I used the fluorophore stainings for IL-2 and the transcription factors c-fos, NFATc2, p65, p-c-jun and FoxP3 (described in Table 1.1), that are included in the time course experiments, to perform a correlation analysis between the concentrations of transcription factors and the frequency of IL-2 producers (Section 3.2.1). By partitioning the available data, an even more detailed analysis and a first qualitative assessment on the question whether different concentrations of one transcription factor affect the probability of IL-2 gene expression as well as the concentration of IL-2 in IL-2 producing cells is presented in Section 3.2.2. A fine-grained even partitioning approach is used to extend the qualitative to a semi-quantitative analysis. This allows a modeling approach investigating how transcription factors affect the probability of IL-2 gene expression in Teff cells (Section 3.2.3) and in Treg cells (Section 3.2.4). In the last section of this chapter, I will address the question which transcription factor is most important for the regulation of IL-2 gene expression.

3.2.1. Median fluorescence intensity of transcription factors fails to predict frequency of IL-2 producers

To investigate whether IL-2 gene expression is affected by the variability of transcription factor concentrations within their endogenous range, I use data from different human donors. I assume that the gene expression varies between human individuals due to differences within their genomes as well as differences in life style and in immunological history but that the regulation of IL-2 gene expression is the same for each donor.

From the observation, that IL-2 gene expression reaches a plateau after four hours (Figure 3.1), it can be concluded that IL-2 gene expression is in a quasi-steady-state at the five hour time point after PMA/Ionomycin stimulation. Also the number of IL-2 producing cells levels off at this time-point. Thus, I only consider the five hour time points data in the correlation analysis in this section. Each data set for every donor and every combination of transcription factors encloses a population of cells, for which I calculated the median fluorescence intensity of FoxP3, and the corresponding second transcription factor listed in Table 1.1. The median fluorescence intensity of the transcription factors are plotted against the frequency of IL-2 producing cells in Figure 3.2.

As FoxP3 is not expressed in Teff cells, the measured fluorescence intensity of the fluorophore

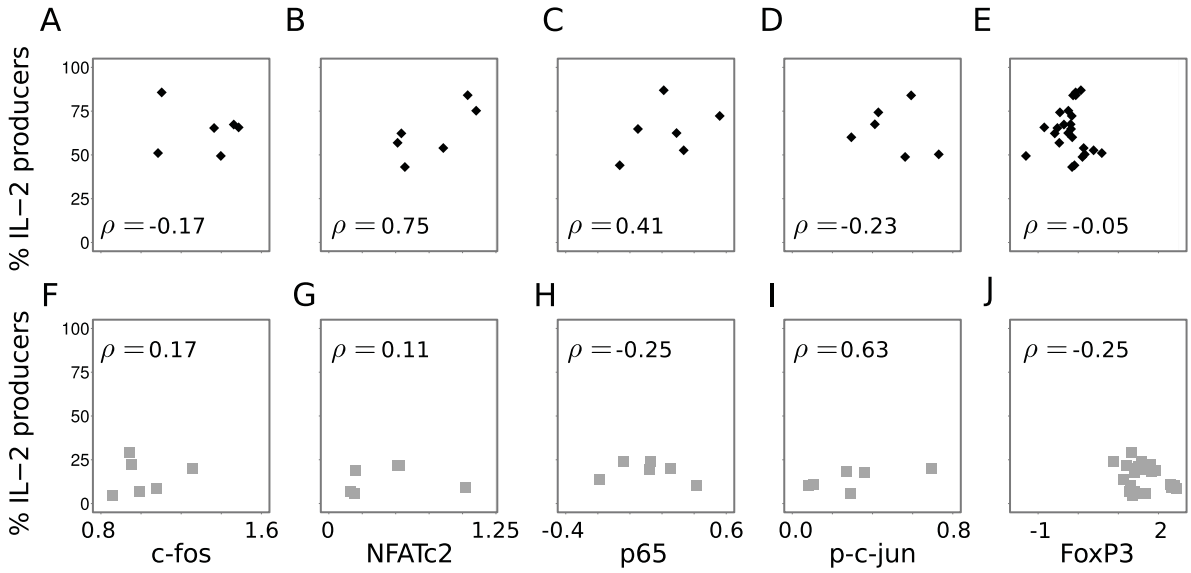

Figure 3.2.: Linear correlation shows small trends among donors. The median values of the normalized fluorescence intensity of the transcription factors are plotted against the frequency of IL-2 producing cells after five hours of stimulation with PMA/Ionomycin. (A)-(E) The black diamonds represent the values of the Teff cells and (F)-(J) the gray squares represent the Treg cells. Pearson's correlation coefficient (ρ) was calculated between median values of data sets from different donors.

phycoerythrin (PE) reflects a background fluorescence rather than a concentration of FoxP3. Thus, the corresponding correlation coefficient should show no existing correlation. Indeed, the correlation coefficient between FoxP3 and IL-2 varies around zero (i.e. no correlation) in Teff cells (Figure 3.2A). In contrast, in Treg cells I expect a negative correlation between FoxP3 expression and the frequency of IL-2 producing cells, because FoxP3 is expressed and represses IL-2 expression. Although Pearson's correlation coefficient shows a negative correlation (Figure 3.2J), this correlation is not significant ($p \geq 0.05$).

With regard to the other measured transcription factors, there seems to be a trend visible in Teff cells (black), namely between the median fluorescence of NFATc2 and IL-2 (Figure 3.2B). This means that a donor with a higher concentration of NFATc2 also has a higher probability of producing IL-2. However, this trend is not significant ($p \geq 0.05$). Also in all other cases, no significant correlation can be detected between transcription factor and the frequency of IL-2 producing cells. In addition, no significant correlations can be detected using the Kendall's rank correlation (not shown).

Taken together, I assumed that the transcription factor abundance as well as the IL-2 gene

expression varies between human individuals, and I expected correlations between transcription factors and the frequency of IL-2 producers if these transcription factors affect gene regulation of IL-2. However, I found that using the median fluorescence intensity is not suitable to detect correlations, despite the fact that the fluorescence intensities of the transcription factors vary between donors in the experimental data.

3.2.2. Transcription factor concentrations correlate with number of IL-2 producers but do not affect IL-2 concentration per cell

Next, I will focus on the endogenous range of the transcription factors within the donors. This is done by dividing the fluorescence intensity of each transcription factor, as a measure of the protein amount per cell, into quartiles in both cell types (Section 2.3). Thereby the endogenous range between donors becomes comparable as only a qualitative partitioning is obtained. Furthermore, this approach allows a reliable estimation of IL-2 producers, because each quartile contains the same number of cells. The distribution of IL-2 expression in each transcription factor quartile is plotted into a histogram for Teff cells (Figure 3.3A-D) and for Treg cells (Figure 3.4A-E) for one representative donor.

The histograms of IL-2 in Teff cells (Figure 3.3A-D) show that the median intensity of the fluorescence IL-2 marker within the IL-2 producers hardly changes between the different quartiles of transcription factors. This is verified by a t-test with adjustment for multiple testing, which shows almost no significant differences in the median fluorescence intensities between the different quartiles (Appendix B, Table B.1). In contrast to the mean fluorescence intensities of IL-2, the number of IL-2 producers in Teff cells strongly correlates with the amounts of c-fos (Figure 3.3E) and NFATc2 (Figure 3.3F), with a Pearson correlation of 0.74 and 0.60, respectively. The different amounts of p65 (Figure 3.3G) and p-c-jun (Figure 3.3H) show a weaker correlation with the number of IL-2 producing cells, with a Pearson correlation of 0.16 and 0.49, respectively. In addition, a t-test with adjustment for multiple testing for the quartiles shows a significant difference between the lowest and the highest quartile for c-fos and NFATc2 but not for p65 and p-c-jun (Appendix B, Table B.2). For example, for c-fos in the lowest quartile are 33.5 $\pm$ 21.5% IL-2 producers detected, whereas in the highest quartile are 80.5 $\pm$ 9.0% IL-2 producers detected. However, the lowest and highest quartile of p65 have similar numbers of IL-2 producers, 56.9 $\pm$ 17.8% and 63.9 $\pm$ 16.7%, respectively.

Similar to Teff cells, in Treg cells the histograms of IL-2 (Figure 3.4A-E) show that the fluorescence intensity of IL-2 within the IL-2 producers is not affected by the amounts of transcription

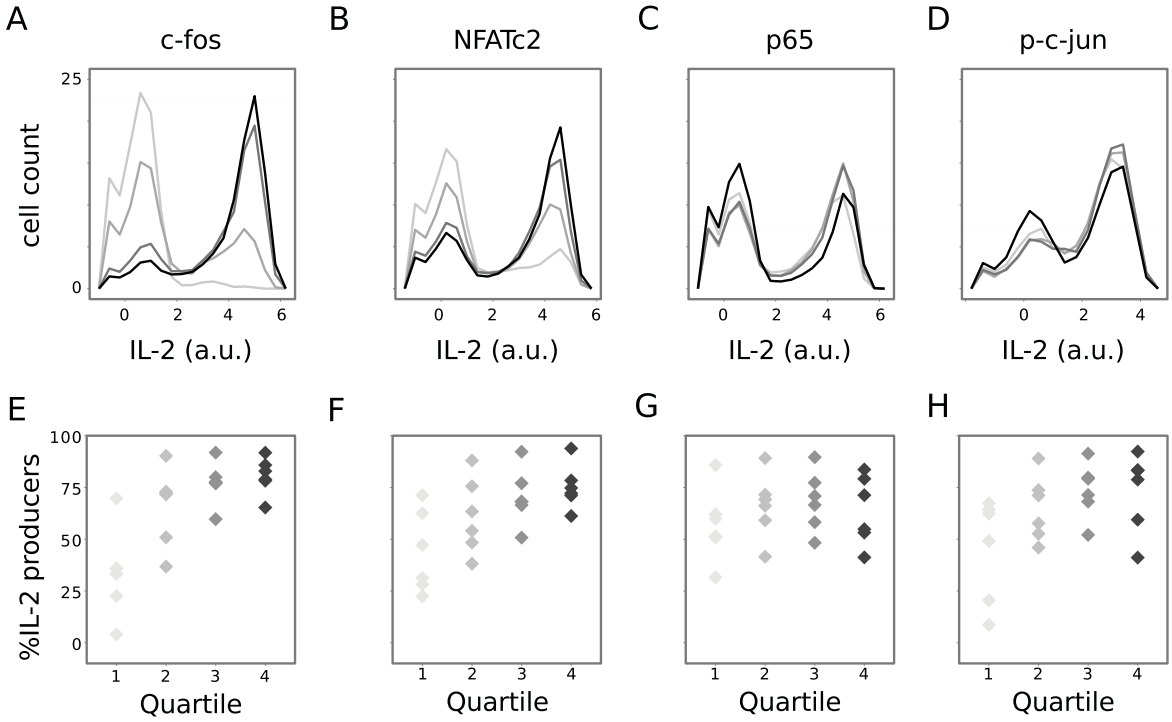

Figure 3.3.: c-fos and NFATc2 concentrations strongly affect the frequencies of IL-2 expressing Teff cells. memory Th cells were stimulated with PMA/Ionomycin for 5 hours, analyzed by flow cytometry, and gated for Teff cells. The fluorescence intensity (FI) distribution of the transcription factors c-fos, NFATc2, p65, and p-c-jun, was divided into quartiles containing the same number of Teff cells. Histogram overlays show the normalized FI of IL-2 for the partitioning of the transcription factor (A) c-fos, (B) NFATc2, (C) p65, and (D) p-c-jun. Data of one representative donor are shown. The percent of IL-2 producing cells are depicted for the partitioning of (E) c-fos, (F) NFATc2, (G) p65, and (H) p-c-jun (n=6). The color code of (A) to (D) corresponds to the color code of the quantiles shown in (E) to (H).

factor. In contrast to Teff cells, not only the fluorescence intensities of the transcription factors c-fos (Figure 3.4F), and NFATc2 (Figure 3.4G), but also the fluorescence intensities of the transcription factors p65 (Figure 3.4H), and p-c-jun (Figure 3.4I) show a strong correlation with the number of IL-2-producing cells ($\rho > 0.82$). FoxP3 itself exhibits a strong negative correlation ($\rho = -0.77$) with the number of IL-2-producing Treg cells (Figure 3.4J). Quartiles with higher fluorescence intensity of FoxP3 (dark gray) have lower numbers of IL-2-producing cells (12.4 $\pm$ 8.0%); while quartiles with low (light gray) fluorescence intensity of FoxP3 per cell exhibit the highest numbers of IL-2 producing cells (28.7 $\pm$ 7.9%).

The investigation of the IL-2 producing Teff cell subpopulation revealed, that the expression

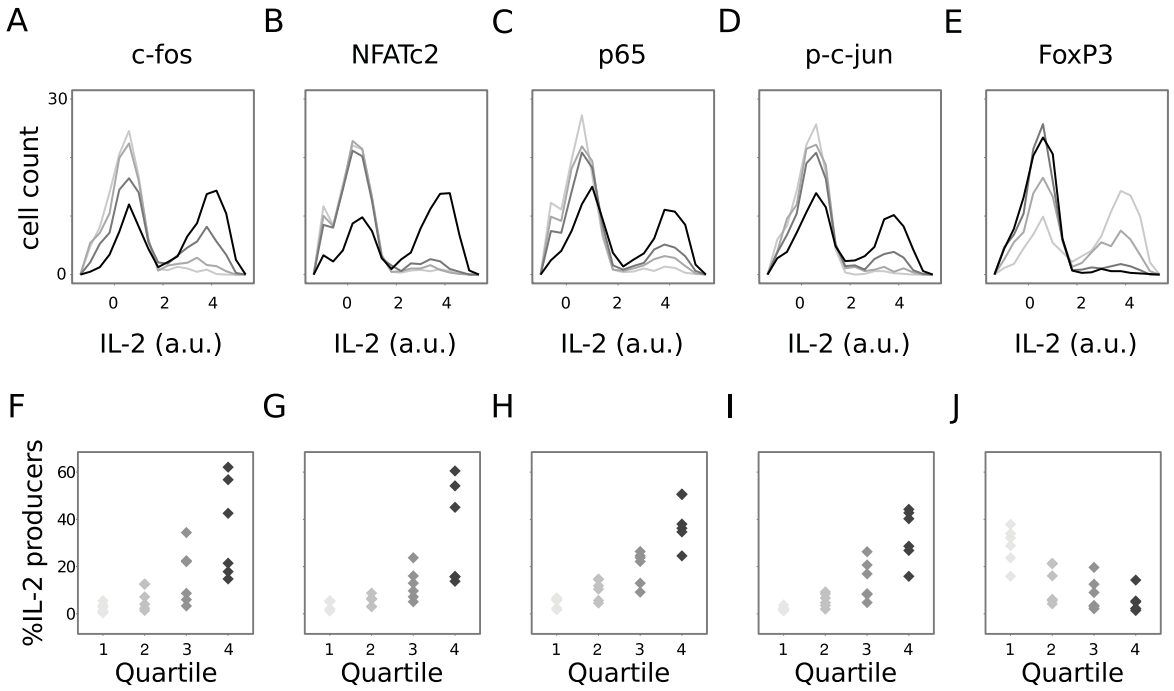

Figure 3.4.: All transcription factors affect the frequencies of IL-2 expressing regulatory Th cells. Memory Th cells were stimulated with PMA/Ionomycin for 5 hours, analyzed by flow cytometry, and gated for Treg cells. The fluorescence intensity (FI) distribution of the transcription factors c-fos, NFATc2, p65, p-c-jun, and FoxP3 was divided into quartiles containing same cell numbers. Histogram overlays show the normalized FI of IL-2 for the partitioning of the transcription factor (A) c-fos, (B) NFATc2, (C) p65, (D) p-c-jun, and (E) FoxP3. Data of one representative donor are shown. The percentage of IL-2 producing cells are depicted for the partitioning of (F) c-fos, (G) NFATc2, (H) p65, (I) p-c-jun, and (J) FoxP3 (n=6). The color code of (A) to (E) corresponds to the color code of the quantiles shown in (F) to (J).

levels of c-fos and NFATc2 determine whether or not a single cell produces IL-2 rather than the amount of IL-2 per cell. In Treg cells, the expression levels of all transcription factors determine whether or not a single cell produces IL-2 but not the amount of IL-2 per cell.

3.2.3. Modeling the probability of inducing IL-2 gene expression by one transcription factor in Teff cells

In the previous section, I have demonstrated that the concentration of transcription factors correlate rather with the frequency of IL-2 producers than the amount of IL-2 in these cells. Furthermore, it has been shown that the rate of IL-2 gene expression remains constant after

initiation of transcription (Smith, 2004; Podtschaske et al., 2007). Therefore, I assume in my modeling approaches that the gene expression of IL-2 is in a steady state and I model the number of IL-2 producers instead of IL-2 expression level as a function of transcription factor concentration (Section 2.7, Figure 2.3).

The analysis in Section 3.2.2 uses a quartile partition, which only allows qualitative statements about a correlation between the transcription factors and IL-2 gene expression. In order to explore the mechanism of regulation in more detail, I now use a fine-grained even partition (Section 2.3) for each transcription factor. As seen in Section 3.2.1 the amounts of transcription factors vary between donors. To make the fluorescence intensities comparable between donors, I normalized and pooled the fluorescence intensities as described in Section 2.2.

In Figure 3.5 the percentage of IL-2 producers in memory Th cells is depicted related to the transformed and normalized fluorescence intensity of the transcription factors c-fos, NFATc2, p65, and p-c-jun (Figure 3.5A-D, respectively). The mean and standard error of the mean of six healthy human donors are indicated with black symbols. Using these data, I estimated the parameters for three models (Section 2.7, Equations 2.11-2.13), which could explain how a transcription factor affects the probability of IL-2 gene expression (Section 2.7). The first model assumes that the IL-2 gene switches between the two states with a fixed probability independent of a transcription factor concentration (independent model). The second model assumes that the IL-2 gene switches between its activated and inactivated state if one transcription factor binds to the DNA (MM model). The third model assumes that the IL-2 gene switches between its two states if more than one transcription factor bind cooperatively to the DNA (Hill model).

Table C.1 shows the estimated parameters for each model and each transcription factor as well as two different measures for the goodness of a fit, being goodness of fit (GoF) and Akaike's information criterion, corrected for sample size (AICc) (explained in Section 2.8). For each of the two measures, a smaller value of goodness of fit (GoF) or AICc indicates a better fit of the model to the given data. The best model for each transcription factor is marked in Table C.1 and plotted as a dark gray line in Figure 3.5.

The best model for c-fos, NFATc2 and p-c-jun is the Hill model with high values for the Hill-coefficient. Although a high Hill-coefficient might imply highly cooperative behavior, a general measure of ultrasensitivity is dependent on the basal activation level as well. For the lowest concentrations of NFATc2 and p-c-jun the frequency of IL-2 producers is around 35% (as indicated by parameter b in Table C.1), which means that even low endogenous concentrations of NFATc2 and p-c-jun do not abolish the probability of IL-2 gene expression

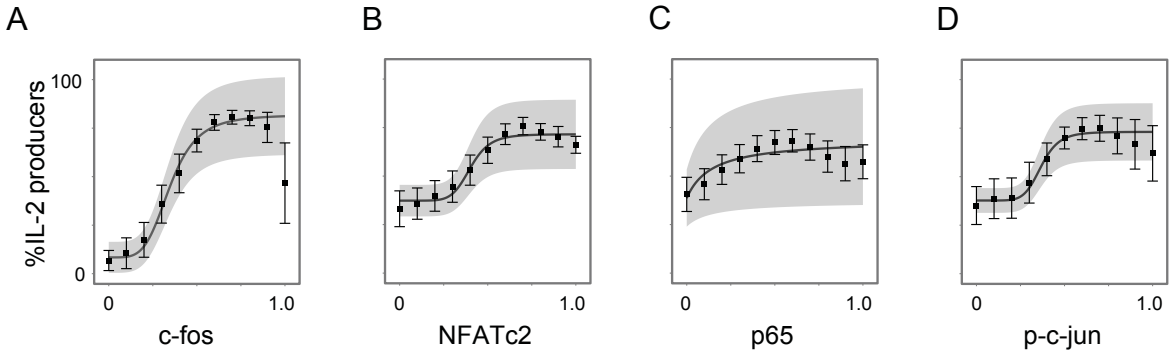

Figure 3.5.: c-fos regulates IL-2 gene expression in a non-linear manner in Teff cells. Human memory Th cells were stimulated with PMA/Ionomycin for 5h, analyzed by flow cytometry and gated for Teff cells. The transformed fluorescence intensities of the transcription factors were divided into 11 bins. The frequency of IL-2 producers were calculated for each bin and the mean and standard error of the mean were plotted (n=6). The best fit of three different models (Section 2.7, Equation 2.11-2.13) is shown for each transcription factor (dark gray line) including the 95% confidence interval (light gray area).

completely in Teff cells. In contrast low endogenous concentrations of c-fos strongly reduces the probability of IL-2 gene expression. In the case of p65, the best model is the Hill model using GoF and the MM model using AICc as quality measures. This may indicate that p65 hardly acts cooperatively on IL-2 gene induction. Similar to NFATc2 and p-c-jun, even for the lowest endogenous concentration of p65 still more than 35% of the Teff cells produce IL-2. These results suggest that the number of IL-2 producers in a population of memory Th cells is critically dependent on the amount of c-fos.

The model prediction that the fraction of IL-2 producers strongly depends in c-fos concentration implies that changing the concentration of c-fos would change the percentage of IL-2 producers. It is possible to inhibit c-fos expression using the MEK inhibitor U0126 (DeSilva et al., 1998). This prevents the activation of ERK and the subsequent gene expression of target genes, which includes the transcription factor c-fos. In the following, I will analyze the affects of U0126 on the gene expression of c-fos and on the probability of IL-2 gene expression.

Teff cells were preincubated for 20 min with different concentrations of U0126 and subsequently stimulated with PMA/Ionomycin for five hours (data kindly provided by Dr. Tobias Scheel). In Figure 3.6A the mean fluorescence intensity of c-fos based on the concentration of U0126 is depicted. The mean fluorescence intensity of c-fos linearly decreases with the logarithmic concentration of U0126 ($R^2 = 0.97$).

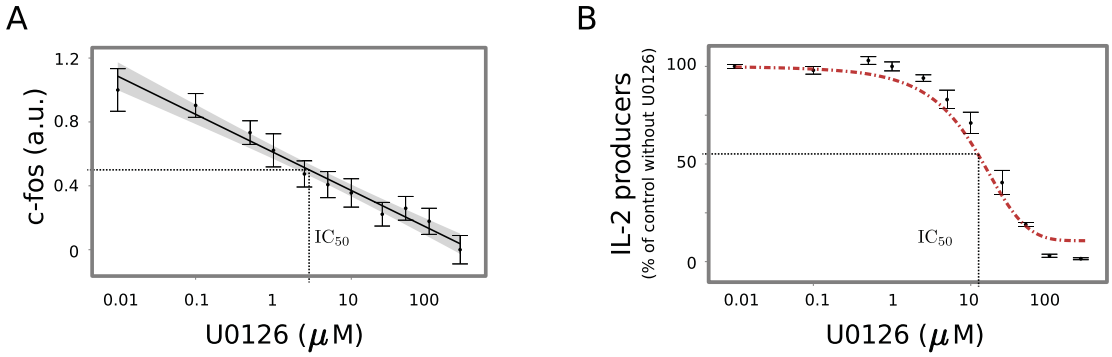

Figure 3.6.: Inhibition of c-fos gene expression decreases percentage of IL-2 producers in a non-linear manner. Human memory Th cells were preincubated with different concentrations of U0126 for 20 min and subsequently stimulated with PMA/Ionomycin for 5 h. Teff cells were gated and analyzed for c-fos and IL-2 expression. (A) Graph depicting the normalized mean c-fos expression at different U0126 concentrations (n=6). The fit for a logarithmic relationship between U0126 and c-fos is indicated by a black line and the 95% confidence interval by the shaded gray area. (B) The prediction of the percentage of IL-2 producers as function of U0126 inhibitor concentration predicted by modeling is shown as red dashed line. The percentage of IL-2 producers in dependency of different concentrations of U0126 from the inhibition experiment are shown as black dots with standard deviation (n=6).

Assuming a logarithmic relationship between c-fos and U0126 with

$$< \text{c-fos} >= a \cdot \log_{10} U0126 + b, \tag{3.1}$$

the parameters of the intercept b and of the slope a can be estimated from the data of Figure 3.6A (Table C.2). The fit of the logarithmic model is depicted as a black line including the 95% confidence interval (Figure 3.6A, gray area). The inhibitory effect of U0126 leads to an half maximal inhibitory concentration (IC_{50}) value of roughly $2.9 \pm 0.9 \mu M$ for c-fos. With this relation and the Hill-like model, I predict the effect of c-fos on the probability of IL-2 gene expression (Figure 3.6B, red dashed line). The model predicts a non-linear decrease in the frequency of IL-2 producers with an IC_{50} of $\approx 12.6 \mu M$. These modeling predictions were than compared to experimental data (Figure 3.6B, black dots). Applying different concentrations of U0126 concentrations to memory Th cells, the number of IL-2 producers, normalized to the number of IL-2 producers in a control data set without inhibition are determined. The mean and the standard error of the mean for six donors are shown. One can see, that the number of IL-2 producers drops from 100% to 0% in a non-linear way even if using a logarithmic scale

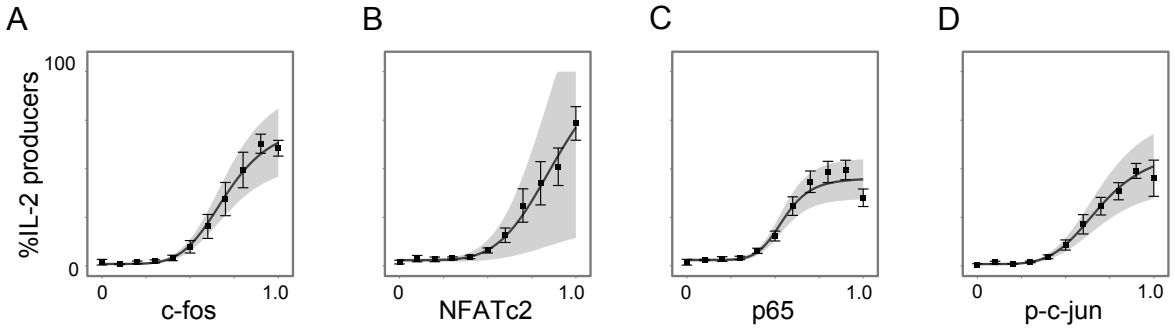

Figure 3.7.: All transcription factors regulate IL-2 gene expression in a non-linear manner in Treg cells. Human memory Th cells were stimulated with PMA/Ionomycin for 5h and analyzed by flow cytometry and gated for Treg cells. The transformed fluorescence intensities of the transcription factors were divided into 11 bins. The frequency of IL-2 producers were calculated for each bin and the mean and standard error of the mean were plotted (n=6). The best fit of three different models (Section 2.7, Equation 2.11-2.13) is shown for each transcription factor (gray line).

for the concentration of the inhibitor U0126 with a good agreement to the prediction. The number of IL-2 producers for high concentrations of U0126, however, are overestimated by the model predictions.

3.2.4. Modeling the probability of inducing IL-2 gene expression by one transcription factor in Treg cells

It has been shown by example (Bendfeldt, 2010), that the fluorescence intensity of all transcription factors is lower in Treg cells compared to Teff cells (analyzed in Appendix C.3). If the regulation of IL-2 gene expression in Treg cells is similar to the regulation in Teff cells, I would expect that the dose response curves are similar to the ones seen in Teff cells (Figure 3.5) only shifted.

Similar to the modeling approach used for Teff cells data of Treg cells were used to model the relation between c-fos, NFATc2, p65, and p-c-jun with the probability of IL-2 gene expression. The percentage of IL-2 producers in Treg cells is depicted related to the transformed and normalized fluorescence intensity of the transcription factors c-fos (Figure 3.7A), NFATc2 (Figure 3.7B), p65 (Figure 3.7C), and p-c-jun (Figure 3.7D). The mean and standard deviation of six human donors are shown. I estimated the parameters of the independent model and the Hill-like model for each of the transcription factors in Treg cells (Table C.3); the fitting of the MM-like model did not converge and is thus not considered. The best model describing the

available data is highlighted in Table C.3 and plotted as a gray line in Figure 3.7 including the 95% confidence interval.

The best model for all transcription factors is the Hill-like model indicating that the switching of the IL-2 gene depends on the concentration of the transcription factors in a non-linear way. This result is in agreement with the qualitative analysis of correlation in Section 3.2.2, where all transcription factors strongly correlate with the probability of IL-2 gene expression. In contrast to the effects seen in Teff cells, for low endogenous concentrations of any of the transcription factors the probability of IL-2 gene expression is now abolished.

3.2.5. Identifying the transcription factor with the highest influence on IL-2 gene expression

In the previous sections I have shown, that and how the different transcription factors c-fos, NFATc2, p65, and p-c-jun affect the probability of IL-2 gene expression. In the following section, I will approach the question which of the transcription factors is the most important one.

In this analysis, I include the measurements from all donors at all time-points stimulated with PMA/Ionomycin. The fluorescence intensities of the transcription factors were normalized into 11 bins each, leading to a two-dimensional grid. For each bin the number of IL-2 producers was calculated. As a measure of importance I use the explained variance in a linear regression model (Section 2.6). The main obstacle for addressing this problem is that the data only allow a comparison of FoxP3 and one other transcription factor. Hence, it is not possible, to obtain a linear regression model of the number of IL-2 producers depending on all transcription factors at once. However, it is feasible to obtain a linear regression model including FoxP3 and one other transcription factor leading to four models using Teff cells data and four models using Treg cells data.

In Figure 3.8 the dispersion measure for FoxP3 vs. the other transcription factors is shown as a scatterplot. The activating transcription factors c-fos, NFATc2, p65, and p-c-jun are color-coded with violet, green, orange, and light-violet, respectively (similar to Figure 1.3). Analyzing the dispersion measure of the transcription factors c-fos, NFATc2, p65, and p-c-jun over time (Figure 3.8A), one can identify three effects. First, the dispersion measure of all these transcription factors increase over time (later time-points are depicted with larger points). This correlates with the increase of IL-2 producers over time (Figure 3.1). Second, each transcription factor can explain up to 80% of the variance of the data in each experiment over time, whereas

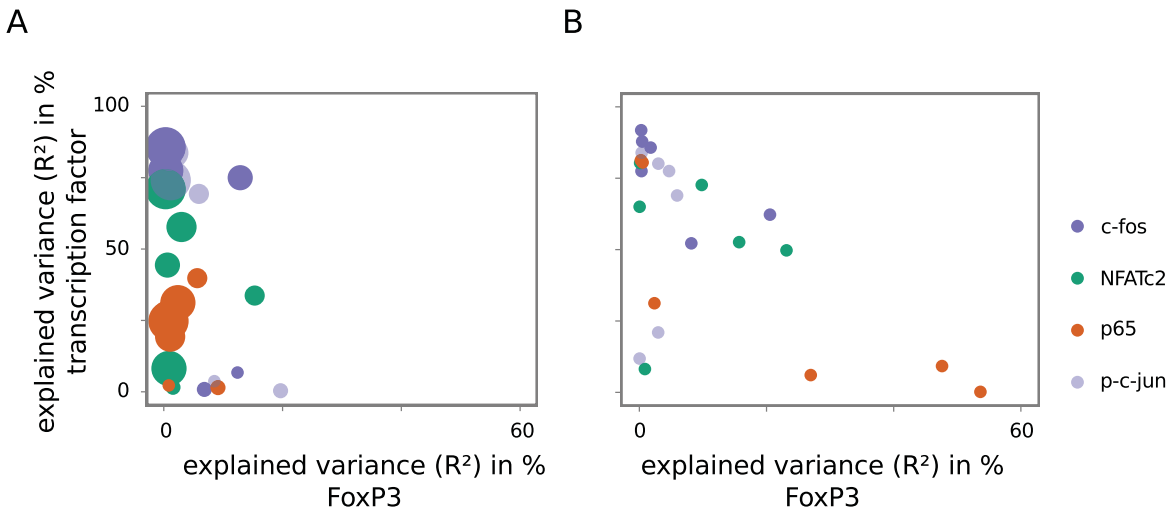

Figure 3.8.: Dispersion measure for all transcription factors in Teff cells. Human memory Th cells were stimulated with PMA/Ionomycin. The cells were harvested at different time-points and co-stained for IL-2, FoxP3, and an additional transcription factor. (A) The dispersion measure for each transcription factor over time in Teff cells for one donor is displayed. Larger points indicate later time-points (Table 2.1). (B) The dispersion measure for each transcription factor across donors in Teff cells after five hours of stimulation is displayed.

FoxP3 explains at most 20%. Third, the transcription factors c-fos and p-c-jun cluster in the top left corner, which means they have the highest dispersion measure in Teff cells over time. In the experiment with CD3/CD28 stimulation similar effects for c-fos and p-c-jun can be seen (Figure D.1).

Comparing the dispersion measure across donors (Figure 3.8B), one can also identify c-fos and p-c-jun as transcription factors with high importance as they are also clustered in the top left corner. Using the dispersion measure, I ranked p-c-jun higher than NFATc2 although they have similar dose response curves (Section 3.2.3). Apparently also FoxP3 has a high effect for some donors in combination with the p65 measurement, which seems to be counterintuitive as FoxP3 is not expressed in Teff cells.

In Treg cells (Figure 3.9), one can also identify the first two effects seen in Teff cells, namely that the dispersion measure of all transcription factors increases over time after stimulation and that each transcription factor can explain up to 80% of the variance of the data in each experiment over time. However, the dispersion measure is not able to identify a transcription factor, which has a generally higher effect than the other transcription factors (Figure 3.9A and 3.9B). The similar dispersion measure of the activating transcription factors might be due

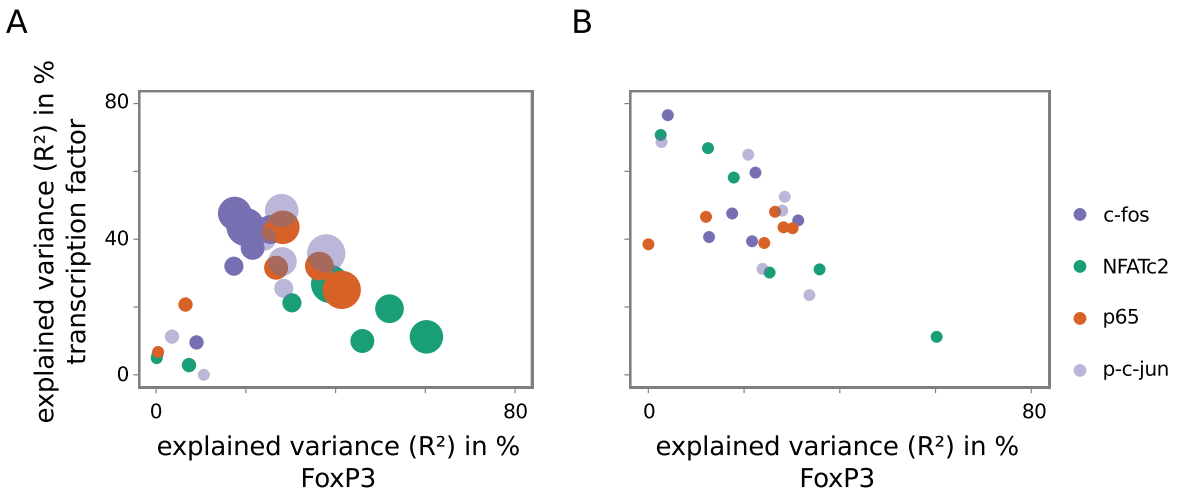

Figure 3.9.: Dispersion measure for all transcription factors in Treg cells. Human memory Th cells were stimulated with PMA/Ionomycin. The cells were harvested at different time-points and co-stained for IL-2, FoxP3, and an additional transcription factor. (A) The dispersion measure for each transcription factor over time in Treg cells for one donor is displayed. Larger points indicate later time-points (Table 2.1). (B) The dispersion measure for each transcription factor across donors in Treg cells after five hours of stimulation is displayed.

to the higher influence of FoxP3, which has an repressive effect and needs to be counteracted by all transcription factors in a concerted manner.

3.3. Investigating the interplay of transcription factors in the regulation of IL-2 expression

It has been shown, that several options for complex formation between the different transcription factors exist (Macián et al., 2001). A well studied example is the transcription factor complex AP-1, which consists mainly of c-fos and p-c-jun (Jain et al., 1992a; Shaulian, 2010). Furthermore, AP-1 and NFAT bind as a complex to the DNA (Macián et al., 2001), enhancing the binding affinity of each other to the DNA. Additionally, the transcriptional repressor FoxP3 is able to form a complex with NFAT using the same binding site as AP-1 and in this way prevents the binding of NFAT to the DNA (Wu et al., 2006).

So far, I have investigated the relation between one transcription factor and the probability of IL-2 gene expression in Teff cells and in Treg cells. In the first section of this chapter, I will concentrate on whether the cooperative effect of NFATc2 and c-fos on DNA binding also leads to a cooperative dose-response in the frequency of IL-2 producers in memory Th cells. In the second section, I will focus on Treg cells and whether FoxP3 also has an inhibitory effect on the frequency of IL-2 producers in relation to the activating transcription factors NFATc2, c-fos, p65, and p-c-jun.

3.3.1. The activation of IL-2 gene expression in memory T helper cells regulated by NFATc2 and c-fos

In this section, I focus on the mutual regulation of IL-2 gene expression through c-fos and NFATc2. Similar to the previous sections, I assume that the gene expression of IL-2 is in a steady state and only the number of cells expressing IL-2 depends on transcription factor concentration. Memory Th cells were stimulated for 5 hours using PMA/Ionomycin and stained for c-fos, NFATc2, and IL-2 (the data were kindly provided by Dr. Tobias Scheel). Normalized fluorescence intensities from three healthy donors were binned into 11 bins for the transcription factors c-fos and NFATc2, creating 121 two-dimensional bins (described in Section 2.3). I calculated the percentage of IL-2 producers for each 2D bin, which had more than three cells. The percentage of IL-2 producers are depicted as a heat map with darker shades of gray indicating higher frequencies of IL-2 producers (Figure 3.10A). The data shows, that high fluorescence intensities of c-fos as well as high fluorescence intensities of NFATc2 imply a high percentage of IL-2 producers. Very few cells contain a combination of high concentrations of NFATc2 and low concentrations of c-fos (Figure 3.10A, lower right) or a combination of low

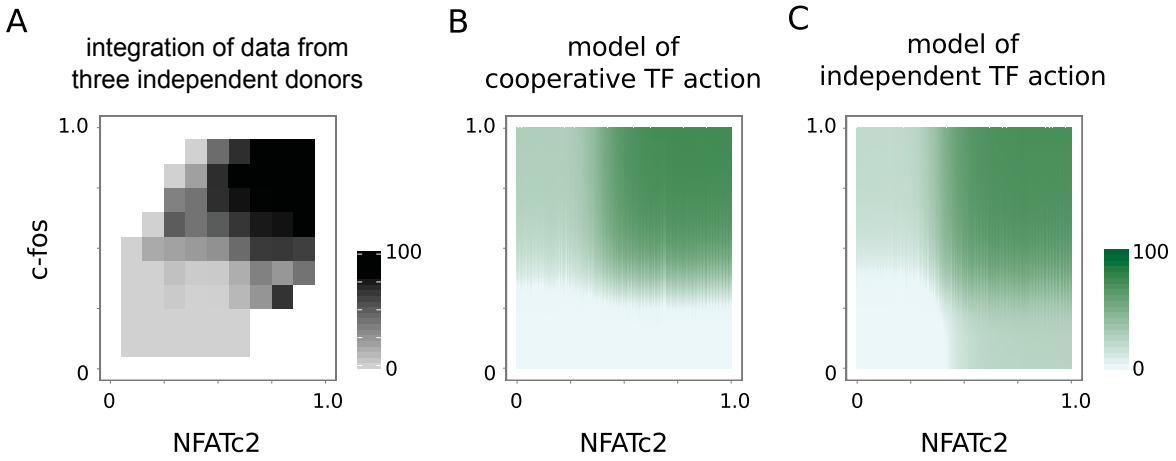

Figure 3.10.: NFATc2 and c-fos act in a cooperative manner in IL-2 production. (A) Human memory Th cells were stimulated with PMA/Ionomycin for 5h and analyzed in parallel for IL-2, c-fos, and NFATc2 expression per cell by flow cytometry. The transformed fluorescence intensity of the transcription factors were each divided into 11 bins. The frequency of IL-2 producers were calculated for each resulting 2D bin and plotted in a heat map showing only bins, which contain more than three cells (n = 3). (B,C) Two different models were fitted to the data, with (B) showing the fit of a model with cooperative regulation of NFATc2 and c-fos to the experimental data and (C) showing the fit of a model with independent regulation.

concentrations of NFATc2 and high concentrations of c-fos (upper left). It is thus not possible to estimate the frequency of IL-2 producers in these regions.

To investigate the dose-response behavior of the frequency of IL-2 producers based on the combination of the transcription factors, I fitted two models (the cooperative model and the independent model, Equation 2.14 and Equation 2.15, respectively) to the heat map data of Figure 3.10A. In the cooperative model, I assume that the number of IL-2 producers is dependent on an concerted action of NFATc2 and c-fos, while in the independent model, an interplay of NFATc2 and c-fos does not play a role (Section 2.7). The estimated parameter sets of the best fits for each of the two models are listed in the supplemental Table E.1. The results of the simulations are shown in Figure 3.10B and 3.10C for the cooperative model and the independent model, respectively.

Both models show an increase in the percentage of IL-2 producers for increasing amounts of NFATc2 and c-fos, which agrees well with the data of Figure 3.10. Also, both models explain the steep increase in the frequency of IL-2 producers along the diagonal from a low concentration of c-fos and NFATc2 to a high concentration of c-fos and NFATc2. The model

of independent transcription factor action shows a lower influence of c-fos compared to the cooperative model, if the concentration of NFATc2 is high (right areas in Figure 3.10B-C). In that case, the frequency of IL-2 producers changes from 50% to 100% for increasing c-fos concentration. In contrast, the model of cooperative transcription factor action shows a steep increase from 0% to 100% in the frequency of IL-2 producers along the concentration of c-fos for high concentrations of NFATc2. Because there are no data available for low concentrations of c-fos combined with high concentrations of NFATc2, it is difficult to distinguish the two models. However, by comparing the goodness of fit, the cooperative model is favored by the AICc measure and the GoF measure over the independent model (Table E.1).

3.3.2. Effects of the inhibitor U0126 in the mutual regulation of IL-2 gene expression by c-fos and NFATc2

The analysis of the previous section indicated that a cooperative model of c-fos and NFATc2 regulating IL-2 gene expression may best describe the available data (Figure 3.10B). The available data (Figure 3.10A) do not allow a clear distinction between a cooperative and independent model of transcription factor concentration. In Section 3.2.3, I have shown that the inhibitor U0126 effects the concentration of c-fos and it may thus be feasible to obtain cells characterized by low c-fos and high NFATc2 levels. This approach may allow to distinguish between the cooperative model and the independent model of transcription factor action.

In this section, I predict the inhibitory effect of U0126 on the mutual regulation of c-fos and NFATc2. It has been shown, that U0126 affects the mean of the c-fos concentration in the population but not the variance, and U0126 does not affect the fluorescence intensities of NFATc2 (Bendfeldt et al., 2012a). Based on these assumptions, I simulated 10^5 cells with each cell having a different concentration of NFATc2 and c-fos. The simulated population of cells is partitioned in 2D bins. For each 2D bin, I predict the frequency of IL-2 producers using the independent model with a concentration of 1 μM, 10 μM, and 100 μM U0126 (Figure 3.11A-C, respectively). As comparison, I also simulated 10^5 cells and predict the frequency of IL-2 producers using the model of cooperative transcription factor action (Figure 3.11D-E).

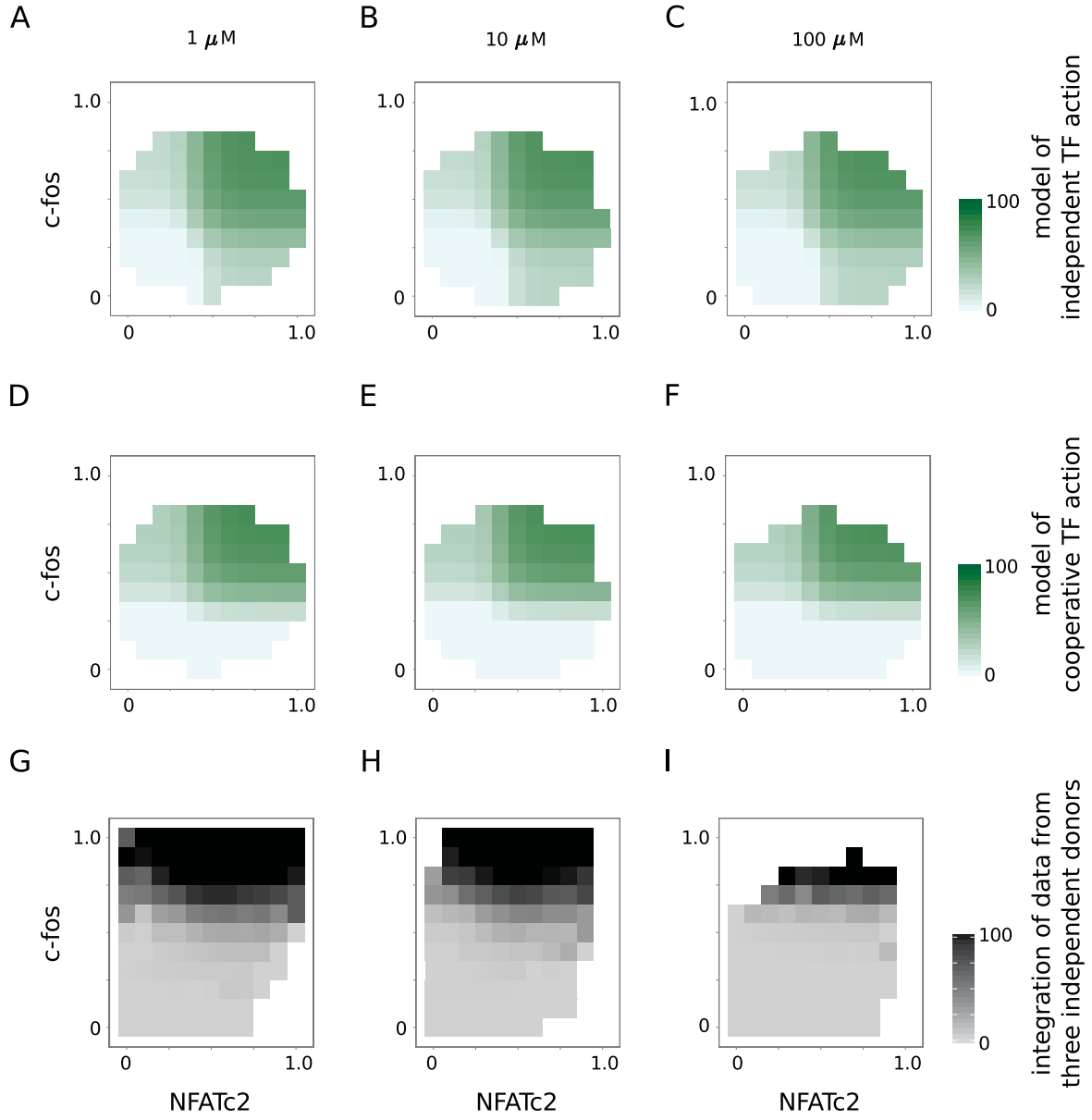

Figure 3.11.: Inhibition of c-fos expression leads to fewer IL-2 producing memory Th cells. The frequency of IL-2 producers are predicted using (A)-(C) the independent activation model and (D)-(F) the cooperative activating model in combination with the model of U0126 inhibition (Equation 3.2.3) for three different concentrations of U0126. (G)-(I) Human memory Th cells were preincubated with three different concentrations of U0126 and than stimulated with PMA/Ionomycin for 5h and analyzed in parallel for IL-2, c-fos, and NFATc2 expression per cell by flow cytometry. The transformed fluorescence intensity of the transcription factors were divided into 121 2D-bins. The frequency of IL-2 producers were calculated for each 2D-bin and plotted as a heat map showing only 2D-bins, which contain more than three cells (n=3): (A, D, G) U0126 = 1 μM, (B, E, H) U0126 = 10 μM, and (C, F, I) U0126 = 100 μM.

All simulations predict, that the shift in the mean c-fos concentration due to the increasing concentration of U0126 leads to less cells having high concentrations of c-fos (white area in the upper part of the heat maps in Figure 3.11A-F) This shift in the distribution of c-fos increases the number of cells having a high NFATc2 concentration and a low c-fos concentration, thus allowing a distinction between the independent model and the cooperative model. As seen in Section 3.3.1, the main difference between the two models is the behavior of the frequency of IL-2 producers for high concentrations of NFATc2 and low concentration of c-fos. For this combination of transcription factor concentrations, the cooperative model predicts no cell can produce IL-2, while the independent model predicts almost 50% IL-2 producers.

To validate this model prediction, I used data from human memory Th cells, which were preincubated with three different concentrations of U0126 ($1\mu M, 10\mu M$, and $100\mu M$). The cells were stimulated for 5 hours and analyzed in parallel for NFATc2, c-fos, and IL-2. The overall frequency of IL-2 producers decreases from 94 % down to 12% of the frequency of IL-2 producers compared to a control sample (as shown in Figure 3.6, Section 3.2.3 and Figure E.1A). Normalized fluorescence intensities from three healthy donors were binned into 11 bins for the transcription factors c-fos and NFATc2, respectively. I calculated the frequency of IL-2 producers for each range of fluorescence intensities, which had more than three cells (Figure 3.11G-I). For higher concentrations of U0126 less cells have high concentration of c-fos (compare Figure 3.11G with Figure 3.11I) as predicted from both models (Figure 3.11A-F). In addition, for high concentrations of NFATc2 but low concentrations of c-fos, the frequency of IL-2 producers is lower than 5% agreeing well with the prediction of the cooperative model and rejecting the independent model.

3.3.3. The inhibition of IL-2 gene expression in regulatory T helper cells regulated by FoxP3

In this thesis so far, the transcription factor FoxP3 has been used as a marker to define Treg cells. However, FoxP3 is a transcription factor, which can also function as a transcriptional repressor of IL-2 gene expression (Marson et al., 2007). In particular, FoxP3 has been shown to bind to NFAT in direct competition to AP-1 (the complex of c-fos and p-c-jun) (Wu et al., 2006). In this section, I will explore to what extend the effect of the transcription factors c-fos, NFATc2, p65, and p-c-jun on the frequency of IL-2 producers (Section 3.2.4) is affected by the transcription factor FoxP3.

In a first approach, I assume that FoxP3 does not influence the regulation of the frequency of

IL-2 producers by the transcription factors c-fos, NFATc2, p65, and p-c-jun. The dependency of the frequency of IL-2 producers on one of these transcription factors can thus be modeled by employing the models for each transcription factor derived in the analysis of Section 2.7, Figure 3.5. The model simulations (Figure 3.12A-D) show an increase of the frequency of IL-2 producers with increasing concentrations of the transcription factors c-fos, NFATc2, p65, and p-c-jun, respectively, but no influence of changing FoxP3 concentration, as expected.

To test this hypothesis, human memory Th cells were stimulated with PMA/Ionomycin and the corresponding Treg cells were partitioned as described in Section 2.3. The frequency of IL-2 producers integrated from six donors are displayed in a heat map for the transcription factors c-fos and p-c-jun, that form the transcription factor complex AP-1 as well as for NFATc2 and p65 (Figure 3.12E - 3.12H) with low frequencies depicted in light gray and high frequencies depicted in dark gray. If no measurements were available, the corresponding bins are colored in white. The data shows, that with increasing fluorescence intensity of c-fos or NFATc2 the frequency of IL-2 producers increases to over 70% (Figure 3.12E and F, respectively). Whereas with increasing fluorescence intensity of p65 or p-c-jun the frequency of IL-2 producers increases up to $\approx$ 60 % (Figure 3.12G and H, respectively). The frequencies of IL-2 producers are anti-correlated with the fluorescence intensity of FoxP3 showing the highest frequency of IL-2 producers in areas of low fluorescence intensity of FoxP3 (lower right area of the heat maps in Figure 3.12E-H). These results suggest that the concentration of c-fos, NFATc2, p65, and p-c-jun have to be strongly increased and FoxP3 reduced in Treg cells to yield high frequencies of IL-2 producers.

The data show, that FoxP3 exerts an inhibitory effect on the frequency of IL-2 producers with respect to all transcription factors. In order to explore the inhibitory effect more, I employ the competition model (Equation 2.16) introduced in Section 2.7. I fit this model to the data shown in Figure 3.12E-H. In Figure 3.12I - L the corresponding model fits for a heuristic inhibition model (Equation 2.16, Section 2.7) are shown. The parameters are available as supplementary material (Table F.1). The model fits show the same increase in the frequency IL-2 producers as seen in the data (Figure 3.12E-H) indicating that a balance of the transcription factors regulates the frequency of IL-2 producers. The estimated values of the model parameter c_2, which reflects the influence of the inhibition by FoxP3, are comparable between the models for the different transcription factors. Thus, the transcription factor FoxP3 has a similar inhibitory effect independent of the choice of the activating transcription factor considered.

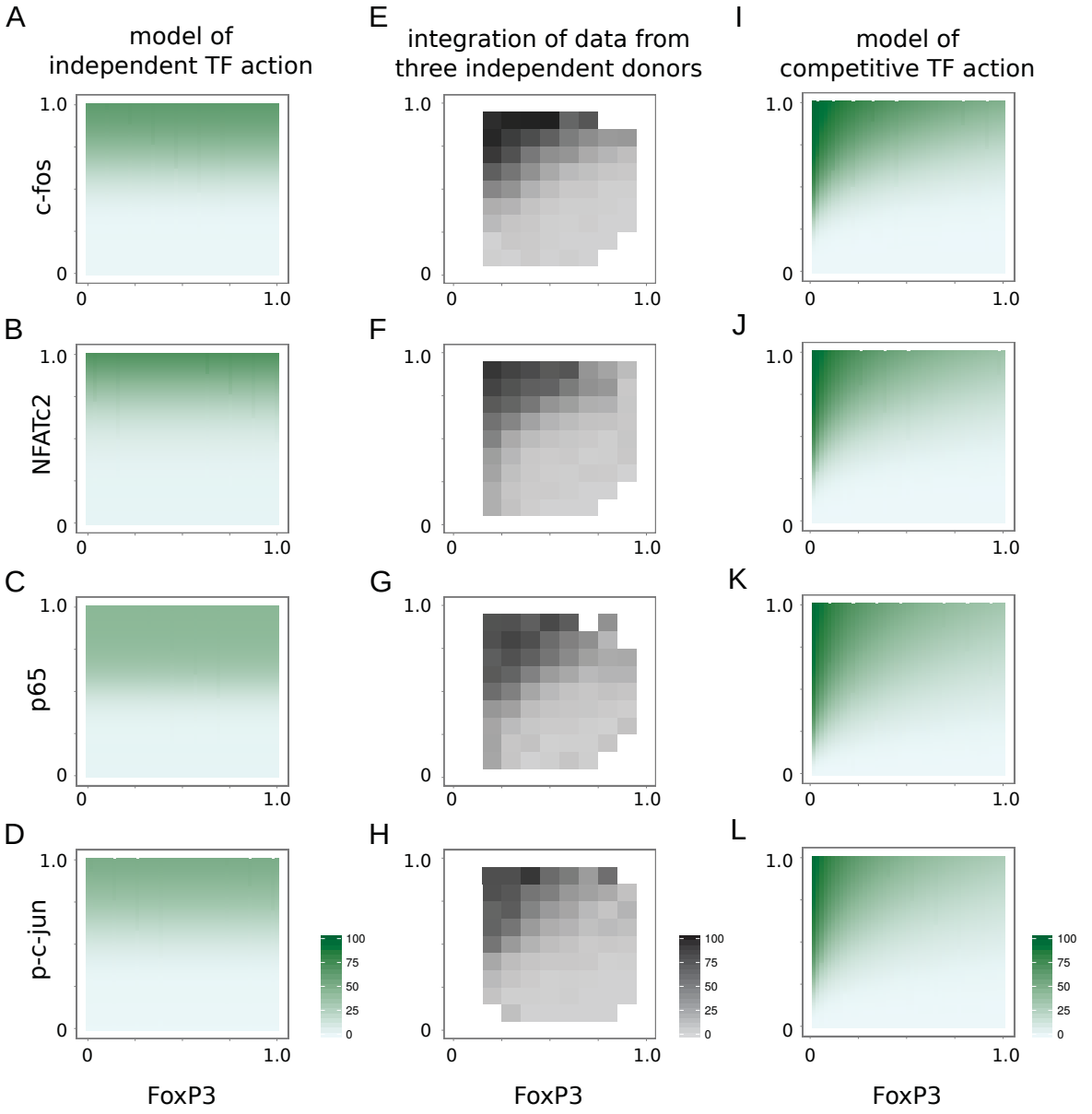

Figure 3.12.: Competitive inhibition of IL-2 gene expression by FoxP3. (A)-(D) Simulation of the models from Section 3.2.4 on a two-dimensional grid for c-fos, NFATc2, p65, and p-c-jun, respectively. The transcription factor FoxP3 exerts no influence on the frequency of the IL-2 producers. (E)-(H) Human memory Th cells were stimulated with PMA/Ionomycin for five hours and analyzed by flow cytometry and gated for Treg cells. The transformed fluorescence intensities of the transcription factors and FoxP3 were divided into 2D-bins. The frequency of IL-2 producers were calculated for each 2D-bin and plotted in a heat map showing only 2D-bins, which contain at least three cells. Data from six different donors were integrated. (I)-(L) The fit of the competitive inhibition model for the data is depicted for each transcription factor using the best parameters.

4. Discussion

I have used the endogenous heterogeneity of transcription factor concentrations (Figure 3.3) to explore how the transcription factors c-fos, NFATc2, p65, and p-c-jun shape the gene expression of the cytokine IL-2 in Teff cells (Figure 3.3 and 3.5) and Treg cells (Figure 3.4 and 3.7). In particular, I focussed on the frequency of IL-2 producers, which can be used as a proxy for the probability that an individual cell expresses IL-2. I identified c-fos as the transcription factor with the strongest effect in Teff cells based on a statistical analysis (Figure 3.8) and mathematical modeling (Figure 3.5). c-fos generates a sigmoidal dose-response curve of the frequency of IL-2 producers, which ranges from 0% up to 70%. Using a mechanistic model of c-fos action, I correctly predicted the effect of inhibiting the IL-2 gene expression by the c-fos inhibitor U0126 (Figure 3.6). I extended my approach from partitioning the distribution of a single transcription factor to partitioning the joint distribution of two transcription factors. By this, I showed that the known cooperative binding of c-fos and NFATc2 to DNA (Macián et al., 2001) also induces a cooperative response for the probability of IL-2 gene expression (Figure 3.10). I could further support this result by predicting the impact of the c-fos inhibitor U0126 using a heuristic model of cooperative c-fos and NFATc2 action. These predictions were validated with experimental data (Figure 3.11). While in Teff cells predominantly c-fos shapes the IL-2 response, in Treg cells all transcription factors seem to have a similar impact as deduced from statistical analysis (Figure 3.9) as well as from their dose response curves (Figure 3.7). The underlying reason could be the increased expression of FoxP3 in Treg cells compared to Teff cells (Figure 2.1), which limits the maximal frequency of IL-2 producers for each of the transcription factors c-fos, NFATc2, p-c-jun, and p65 (Figure 3.12). The inhibition of the activating effect of NFAT and AP-1 (c-fos/p-c-jun complex) by high FoxP3 concentrations has already been known and a potential molecular mechanism of action has been reported, in which Foxp3 competes with AP-1 for NFAT binding and thereby represses IL-2 expression (Wu et al., 2006). Interestingly, my analysis also proposes an inhibitory influence of FoxP3 on the activation of IL-2 expression by p65. This novel finding may indicate that NFAT, AP-1 and NF-κB together form a larger complex to activate IL-2 gene expression and this larger complex

is perturbed by FoxP3.

After having demonstrated that c-fos has the strongest regulatory impact in Teff cells, I inferred how perturbations of c-fos expression by the inhibitor U0126 would affect IL-2 producer frequency in Teff cells. Using my mechanistic model of c-fos dependent activation, I predicted how inhibition of c-fos decreases the frequency of IL-2 producers in Teff cells and estimated an IC_{50} value of $\approx 12.6\mu M$. Using the data from inhibition experiments, I could verify these predictions (Figure 3.6). Furthermore, I estimated an U0126 dependent IC_{50} of $\approx 2.9\mu M$ for the expression of c-fos but could not verify this with data from the literature. Since U0126 is actually an inhibitor of the mitogen-activated extracellular-regulated kinase (MEK), application of U0126 leads to a reduced activity of extracellular-signal-regulated kinase (ERK) followed by the reduced expression of c-fos, which are both downstream of MEK in the MAPK cascade. U0126 has been experimentally characterized for its inhibitory effects on MEK and ERK revealing IC_{50} value for MEK and ERK has been reported to be $\approx 0.07\mu M$ (Favata et al., 1998) and $\approx 0.2\mu M$ (Scherle et al., 1998), respectively. Comparing the IC_{50} values, I observe an increasing trend of the IC_{50} values from MEK via ERK and c-fos to the frequency of IL-2 producers. On the basis of this observed trend, I would expect the IC_{50} value of U0126 on IL-2 mRNA expression to be in the range of $2.9\mu M$ to $12.6\mu M$. Searching the literature, I found a value of $\approx 1.4\mu M$ published for U0126 treatment of Jurkat cells (Favata et al., 1998), which lies a bit below my expectation. This discrepancy may be explained by the choice of different cell types in the experiments, i.e. cells of the Jurkat cell line in the case of the published value compared to human *ex vivo* Teff cells in my study. A follow-up experiment measuring IL-2 mRNA concentration for different concentrations of U0126 in human *ex vivo* Teff cells can clarify this issue.

As my minimal model can correctly predict how inhibiting the gene expression of a transcription factor on the example of c-fos, the question arises how the models would predict effects on overexpressing transcription factor concentration. The data shows that up to 70% of the Teff cells can produce IL-2 while only 15% of the Treg cells express IL-2 (Figure 3.1). The simulations of my mechanistic models predict that the frequency of IL-2 producers is increased up to 80% in a subpopulation of Teff cells with increased concentration of c-fos (Figure 3.5). This indicates that the overexpression c-fos in Teff cells may lead to an increase in the frequency of IL-2 producers. In Treg cells, the simulations predict a saturation of the frequency of IL-2 producers for increasing concentrations of p65 (45%) and c-fos and p-c-jun (70% in both cases) (Figure 3.7). However, in the case of NFATc2 no saturation is shown (Figure 3.7B). This

implies that an overexpression of NFATc2 in Treg cells may increase the frequency of IL-2 producers even further than 80%, which are seen in the subpopulation with the highest NFATc2 concentration.

It might be difficult to perform the suggested overexpression experiments in human primary T-cells. However, to further validate the predictive power of the minimal models, one could also ask how many cells which produced IL-2 after one stimulation would do so after a second stimulation. Assuming that the IL-2 producers do not change their transcription factor concentrations, a simple hypothesis would be that all cells express IL-2 again if restimulated. However, using my mechanistic models I would expect at most 80% IL-2 producers in a heterogeneous population as one can see a saturation for all transcription factors. Therefore, a restimulation experiment would be an legitimate way to validate my model. To address this question experimentally one could use a secretion assay, where IL-2 is detected directly at a cells' membrane. After that, one can sort IL-2 producers using a secondary antibody and fluorescent-activated cell sorting and subsequently the IL-2 producers can be taken into a new cell culture again for performing a restimulation experiment.

When partitioning the endogenous distribution of the transcription factors to explore the regulation of IL-2 gene expression, I encountered a sigmoidal response as a major type of response behavior in Teff cells as well as in Treg cells. This steep increase in the probability of IL-2 gene expression in response to c-fos concentration in Teff cells, as well as for all transcription factors in Treg cells indicates a threshold-like response in the activation of IL-2 gene expression. These threshold-like responses seem to be a common feature in the immune system. A threshold-like response has also been discussed for the response of Erk1 in cytotoxic T-cells (Feinerman et al., 2008; Germain et al., 2011). Another example is the T-cell receptor, where the effect of multiple activated TCR adds up to eventually reaching a threshold required for the activation of an immunological response (Rachmilewitz and Lanzavecchia, 2002). These threshold like responses might establish a general fail-safe measure to ensure an appropriate response in the processes of the immune system. This means, that a response is suppressed until a critical threshold is passed. After passing this critical threshold a full response is induced. This sharp distinction between no and full response is referred to as kinetic proofreading (revisited in (Germain et al., 2011)).

The time-course experiments presented in this thesis show IL-2 gene expression two hours after stimulation with PMA/Ionomycin. IL-2 gene expression immediately displays a bimodal behavior as previously reported for the single time-point after five hours of stimulation

(Podtschaske et al., 2007). I demonstrated, that this bimodality occurs independent of the time of blocking IL-2 secretion with Brefeldin A, indicating that bimodality is an intrinsic feature of IL-2 gene regulation rather than a consequence of the experimental setup. Although the measured time-series give an idea how a population changes over time it is not possible to track single cells over time. A correlation between the concentrations of the transcription factors and the expression of IL-2 can only be made at one time-point. Therefore, I concentrated on modeling the relation between the transcription factors and IL-2 at five hours after stimulation, when the frequency of IL-2 producers saturated. By using single live cell measurements one might relate how changes in expression of transcription factors over time affects the gene expression of IL-2 at later time points. This is especially of interest as expression of FoxP3 seems often to be a transient event (O'Shea and Paul, 2010). That means, that cells, which have been classified as Treg cells in my thesis based on their FoxP3, expression do not necessarily express FoxP3 all the time. Since the transient FoxP3 gene expression is described in the context of T helper cell differentiation, which is on a longer time-scale than the experimental data used in my thesis, these changes in FoxP3 expression can be neglected.

To identify the impact of the transcription factors on the frequency of IL-2 producers I have used the dispersion measure in addition to mechanistic models. The dispersion measure dissects the explained variance R^2 in a linear regression model and to my knowledge, this measure has not yet been used to analyze effects based on flow cytometry data. For each data set, the dispersion measure is calculated for FoxP3 and an additional transcription factor with respect to the frequency of IL-2 producers. To be able to compare different data sets, I used the fact that in each data set similar dispersion measures for FoxP3 were observed. This approach was necessary, because not all transcription factors could be measured simultaneously in one data set, which may be considered in follow-up experiments. In principle, up to 18 different proteins are detectable by flow cytometry (Ornatsky et al., 2010). The dispersion measure, I used for three-dimensional data, is extendable for higher dimensional data, only limited by the combinatorial explosion for the linear regression models which need to be fitted. However, the dispersion measure is not only suitable for data sets, where the measured proteins are correlated, it also extends the idea of using flow cytometric measurements for classification to predicting the impact of several proteins based on a linear regression model.

A frequently used approach for modeling the regulation of gene expression by transcription factors is thermodynamic modeling (Bintu et al., 2005a,b), which takes into account binding energies for each transcription factor to its available binding sites on a promoter and each

possible transcription factor interaction. In the case of the IL-2 promoter, this approach would lead to a high-dimensional parameter space due to the regulation by the four transcription factors c-fos, NFATc2, p65, and p-c-jun. To estimate parameters for such a detailed model, co-stainings with all transcription factors as well as mutating specific binding sites would be necessary, to gain knowledge about the interactions of all transcription factors and the IL-2 promoter. With this approach, I expect one could further explore how FoxP3 inhibits the effect of p65 (Figure 3.12). However, the available data do not allow to use a thermodynamic modeling approach of the IL-2 promoter.

With the mechanistic models I could show the nonlinear effect of transcription factor concentration in Teff cells (Figure 3.5) as well as in Treg cells (Figure 3.7) in their endogenous range. For the applied concentration of PMA/Ionomycin the transcription factors c-fos, NFATc2, p65, and p-c-jun are fully activated (Bendfeldt, 2010). Thus the range of expressed transcription factor is equivalent to the range of activated transcription factor. *In vivo*, T cells encounter antigens with different avidity, which is accumulated strength of multiple affinities, activating the different transcription factors to varying degrees within a population (Kemp et al., 2007). To be able to translate the models, which are based on *ex vivo* experiments, to predict IL-2 expression *in vivo* my models need to be expanded to include the addition of a regulatory layer of transcription factor expression and activation, as for inhibition of the gene expression of the NFAT isoform NFATc1 by FoxP3 (Torgerson et al., 2009). Including the regulation of the transcription factors in a model can help to account for the effects of inhibitors, such as CsA affecting NFATc2, in a more precise way.

With additional antibodies and improved flow cytometry techniques it might be possible not only to measure the influence of additional transcription factors on the gene expression of IL-2 but also to measure these effects within one experimental setup. Additional transcription factors could include cAMP-responsive element modulator (CREM) and cAMP response element-binding protein (CREB), which seem to play a role in the autoimmune disease systemic lupus erythematosus (SLE), where the levels of IL-2 are markedly decreased (Gómez-Martín et al., 2009). In SLE patients altered gene expression has been found for CREM (increased levels) and CREB (decreased levels), which function as activator and repressor of IL-2 gene expression, respectively (Tenbrock and Tsokos, 2004). My minimal models as well as my statistical analysis are a useful tool to explore the aberrant regulation of IL-2 expression in SLE patients.

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A. Derivation of the differential equations for transcription factor models

For the regulation of IL-2 gene expression, I assume that transcription factors regulate only whether IL-2 is expressed and not how much. Thus the IL-2 gene switches between two states: a state in which transcription does not take place (inactive state) and a state in which the polymerase binds to the transcription start site and transcription is possible (active state). If the switching does not depend on the binding of any transcription factor, than the probability of the gene to be in either state can be modeled using the activation rate k_1 and the inactivation rate k_2 (Figure 2.3A) with

$$\frac{d\text{active}}{dt} = k_1 \cdot \text{inactive} - k_2 \cdot \text{active} \tag{A.1}$$

In addition, there is a conservation relation

$$\text{active} + \text{inactive} = 1, \tag{A.2}$$

which means that the individual probabilities of the two states sum up to one and that the probability of IL-2 gene expression is a probability distribution.
In a steady-state the probability of the IL-2 gene to be in an active state is

$$0 = k_1 \cdot \text{inactive} - k_2 \cdot \text{active} \tag{A.3}$$

$$0 = k_1 \cdot (1 - \text{active}) - k_2 \cdot \text{active} \tag{A.4}$$

$$(k_1 + k_2) \cdot \text{active} = k_1 \tag{A.5}$$

$$\text{active} = \frac{k_1}{k_1 + k_2} = b \tag{A.6}$$

In the case that the switching depends on the binding of one transcription factor (Figure 2.3B),

the differential equation for the system is

$$\frac{d\text{active}}{dt} = k_1 \cdot \text{TF} \cdot \text{inactive} - k_2 \cdot \text{active} \tag{A.7}$$

Using the same conservation relation as above (Equation A.2) the steady-state of the probability of the IL-2 gene looks as follows:

$$0 = k_1 \cdot \text{TF} \cdot \text{inactive} - k_2 \cdot \text{active} \tag{A.8}$$

$$0 = k_1 \cdot \text{TF} \cdot (1 - \text{active}) - k_2 \cdot \text{active} \tag{A.9}$$

$$(k_1 \cdot \text{TF} + k_2) \cdot \text{active} = k_1 \cdot \text{TF} \tag{A.10}$$

$$\text{active} = \frac{k_1 \cdot \text{TF}}{k_1 \cdot \text{TF} + k_2} \tag{A.11}$$

$$\text{active} = \frac{\text{TF}}{\text{TF} + \frac{k_2}{k_1}} \tag{A.12}$$

$$\text{active} = \frac{\text{TF}}{\text{TF} + c} \tag{A.13}$$

The last equation has two properties, which I do not observe in the data. First

$$\text{active}(\text{TF} = 0) = 0 \tag{A.14}$$

and second

$$\lim_{\text{TF}\to\infty} \text{active} = 1 \tag{A.15}$$

To change this I allow two scaling parameters a and b in the final equation

$$\text{active} = \frac{a \cdot \text{TF}}{\text{TF} + c} + b \tag{A.16}$$

In the last case, when one transcription factor has to bind at multiple sites, the differential equation system is

$$\frac{d\text{active}}{dt} = k_1 \cdot \text{TF}^n \cdot \text{inactive} - k_2 \cdot \text{active} \tag{A.17}$$

The derivation of the steady-state follows the same principles as above and leads to the following equation without scaling

$$\text{active} = \frac{\text{TF}^n}{\text{TF}^n + c} \tag{A.18}$$

and with scaling

$$\text{active} = \frac{a \cdot \text{TF}^n}{\text{TF}^n + c} + b. \tag{A.19}$$

B. Pairwise t-test for effect of transcription factor concentration on median fluorescence intensity of IL-2 and on number of IL-2 producers

Table B.1.: Pairwise comparisons between the median fluorescence intensity of IL-2 producers in the different quartiles as investigated in Section 3.2.2 using t-tests with pooled standard deviations for the different transcription factors. Numbers in bold indicate quantiles as given in Figure 3.3E-H and in Figure 3.4F-J. The p-values were adjusted for multiple testing using the Holm-method. p-values below 0.05 are considered significant.

	Teff cells				Treg cells		
			c-fos				
	1	**2**	**3**		**1**	**2**	**3**
2	0.99	-	-	**2**	0.1464	-	-
3	0.54	1.00	-	**3**	0.0238	0.7221	-
4	0.42	1.00	1.00	**4**	0.0047	0.3082	0.7221
			NFATc2				
	1	**2**	**3**		**1**	**2**	**3**
2	1.00	-	-	**2**	1.00	-	-
3	1.00	1.00	-	**3**	1.00	1.00	-
4	1.00	1.00	1.00	**4**	1.00	1.00	1.00
			p65				
	1	**2**	**3**		**1**	**2**	**3**
2	1.00	-	-	**2**	1.00	-	-
3	1.00	1.00	-	**3**	1.00	1.00	-

Continued on next page

Table B.1 – continued from previous page

	Teff cells				Treg cells		
4	1.00	1.00	1.00	**4**	1.00	1.00	1.00
				p-c-jun			
	1	**2**	**3**		**1**	**2**	**3**
2	1.00	-	-	**2**	1.00	-	-
3	1.00	1.00	-	**3**	1.00	1.00	-
4	1.00	1.00	1.00	**4**	0.56	1.00	1.00
				FoxP3			
					1	**2**	**3**
				2	0.3915	-	-
				3	0.0572	0.3915	-
				4	0.0005	0.0221	0.3329

Table B.2.: Pairwise comparisons between the number of IL-2 producers in the different quartiles as investigated in Section 3.2.2 using t-tests with pooled standard deviations for the different transcription factors. Numbers in bold indicate quantiles as given in Figure 3.3E-H and in Figure 3.4F-J. The p-values were adjusted for multiple testing using the Holm-method. p-values below 0.05 are considered significant.

	Teff cells				Treg cells		
				c-fos			
	1	**2**	**3**		**1**	**2**	**3**
2	0.0083	-	-	**2**	0.5569	-	-
3	0.0006	0.4495	-	**3**	0.2113	0.4077	-
4	0.0003	0.36902	0.7245	**4**	0.0009	0.0030	0.0494
				NFATc2			
	1	**2**	**3**		**1**	**2**	**3**
2	0.293	-	-	**2**	0.7563	-	-
3	0.046	0.666	-	**3**	0.5914	0.6403	-
4	0.017	0.444	0.666	**4**	0.0009	0.0016	0.0136

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Table B.2 – continued from previous page

	Teff cells				Treg cells		
	p65						
	1	**2**	**3**		**1**	**2**	**3**
2	1.00	-	-	**2**	0.1722	-	-
3	1.00	1.00	-	**3**	0.0024	0.0406	-
4	1.00	1.00	1.00	**4**	1.6e-07	2.0e-06	0.0004
	p-c-jun						
	1	**2**	**3**		**1**	**2**	**3**
2	0.318	-	-	**2**	0.3989	-	-
3	0.093	1.000	-	**3**	0.02552	0.1062	-
4	0.093	1.000	1.000	**4**	2.4e-06	1.2e-05	0.0009
	FoxP3						
					1	**2**	**3**
				2	0.00268	-	-
				3	0.00032	0.64702	-
				4	6.3e-05	0.25548	0.64702

C. Parameter estimation for single transcription factor models

C.1. Parameter estimation for dose response models in Teff cells

Table C.1.: Parameter estimation for dose response models in Teff cells. Parameters and statistical analysis of three models (Equations 2.11-2.13 in Section 2.7) describing the dependency of frequency of IL-2 producers on transcription factor concentration. The best model for each transcription factor is shown in Figure 3.5A-D.

model	parameter				goodness of fit measures		
	a	b	c	n	GoF	$AICc$	$F-test$
				c-fos			
independent		0.628			25.89	37.19	/
MM-like	1.59	0.0198	0.779		2.36	15.39	0.00003
Hill-like	0.73	0.0843	0.0086	4.46	0.62	4.50	0.00188
				NFATc2			
independent		0.640			5.54	20.24	/
MM-like	0.709	0.265	0.496		1.38	9.50	0.00160
Hill-like	0.342	0.374	0.0013	7.31	0.52	2.43	0.00673
				NF-κB			
independent		0.598			1.34	4.61	/
MM-like	0.304	0.387	0.145		0.72	-0.83	0.010935
Hill-like	0.211	0.429	0.0007	4.519	0.39	-0.71	0.08124
				p-c-jun			
independent		0.631			3.15	14.03	/
MM-like	0.778	0.299	0.591		0.77	3.08	0.00147
Hill-like	0.354	0.377	0.0007	7.265	0.20	-8.00	0.00177

Table C.2.: Parameter estimation for inhibition of c-fos by U0126 in memory Th cells. A logarithmic model of the form $< \text{c-fos} >= a \cdot \log_{10} U0126 + b$ was used for fitting.

a	b	adjusted R^2	p-value
-0.238 ± 0.013	0.609 ± 0.018	0.97	$2.29 \cdot 10^{-08}$

Table C.3.: Parameter estimation for dose response models in Treg cells. Parameters and statistical analysis of three models (Equations 2.11-2.13 in Section 2.7) describing the dependency of frequency of IL-2 producers on transcription factor concentration. The best model for each transcription factor is shown in Figure 3.5E-H.

model	parameter				goodness of fit measures		
	a	b	c	n	GoF	$AICc$	$F-test$
c-fos							
independent		0.014			44.06	43.04	/
Hill-like	0.722	0.0098	0.157	5.35	0.61	4.21	$2.05 \cdot 10^{-07}$
NFATc2							
independent		0.045			13.26	29.83	/
Hill-like	1.12	0.0292	0.639	4.98	0.36	−1.59	$2.16 \cdot 10^{-06}$
NF-κB							
independent		0.039			31.75	39.43	/
Hill-like	0.421	0.0303	0.0144	7.06	1.35	12.97	$1.04 \cdot 10^{-05}$
p-c-jun							
independent		0.013			33.84	40.13	/
Hill-like	0.59	0.009	0.174	4.81	0.48	1.59	$2.24 \cdot 10^{-07}$

Table C.4.: Paired t-test of median fluorescence intensities of transcription factors in Teff cells and Treg cells. A one-sided t-test was used with the alternative hypothesis that the medians of a transcription factor in Teff cells are greater than the medians of a transcription factor in Treg cells.

transcription factor	Mean of differences	p-value
c-fos	0.3039	0.0039
NFATc2	0.3335	0.0010
p65	0.1147	0.0003
p-c-jun	0.2031	0.0078

C.2. Parameter estimation for dose response models in Treg cells

C.3. Comparison of fluorescence intensities of transcription factors in Teff cells and Treg cells

It has been shown by example (Bendfeldt, 2010), that the fluorescence intensity of all transcription factors is lower in Treg cells compared to Teff cells (Figure C.1A-D). The density of the fluorescence intensity in Teff cells (black) and Treg cells (gray) of one representative donor are depicted for c-fos, NFATc2, p65, and p-c-jun, respectively. The distribution of each transcription factor in Teff cells has higher fluorescence intensities than the distribution of the same transcription factor in Treg cells. For each donor, I compared the two subpopulations with a Kolmogorov-Smirnov-test. In each case the null-hypothesis, that the population in Teff cells and in Treg cells stem from the same distribution can be rejected for all donors and all transcription factors ($p < 0.05$). In addition, the medians from each donor are lower in Treg cells compared to Teff cells (Figure C.1E-H) based on the paired t-test (Table C.4) verifying earlier results.

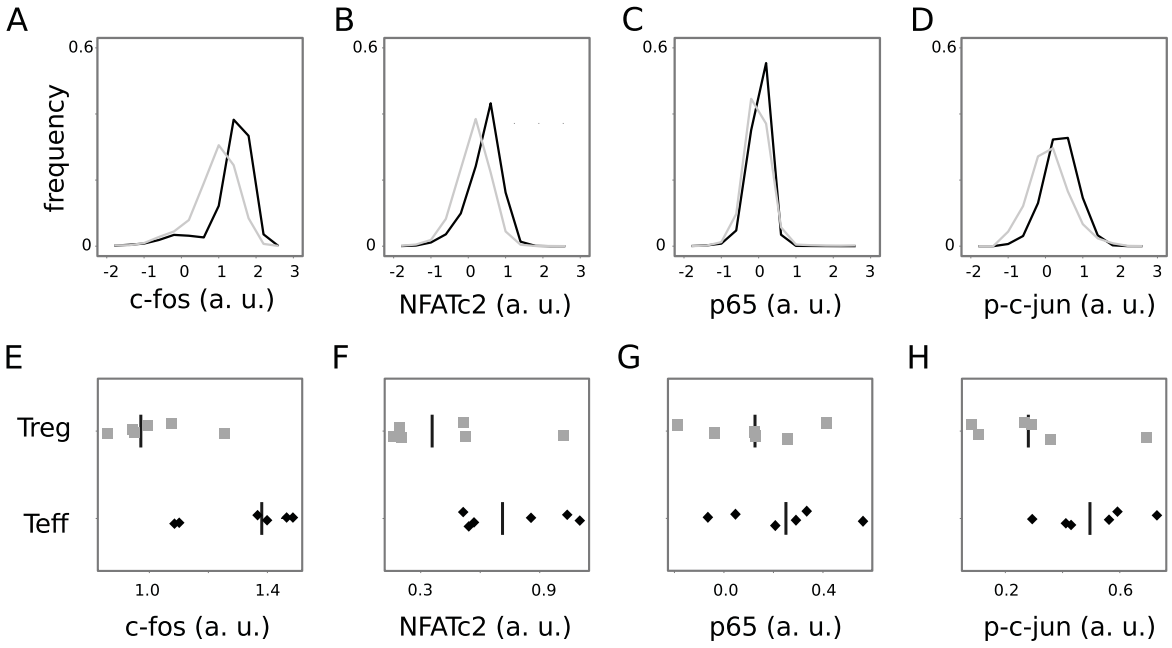

Figure C.1.: The fluorescence intensities of NFATc2, c-fos, NF-κB and c-jun expression levels are lower in Treg cells compared to Teff cells. Memory Th cells were stimulated with PMA/Ionomycin for 5h and analyzed in parallel for IL-2, FoxP3, and one activating transcription factor per cell by flow cytometry. The cells were gated for Teff cells and Treg cells. For each transcription factor the density of the fluorescence intensity in Teff cells (black) and Treg cells (gray) of one representative donor are depicted. In the inset the median fluorescence intensities for each transcription factor in Teff cells (black diamonds) and Treg cells (gray squares) are shown.

D. Calculating the dispersion measure for cells stimulated with CD3/CD28

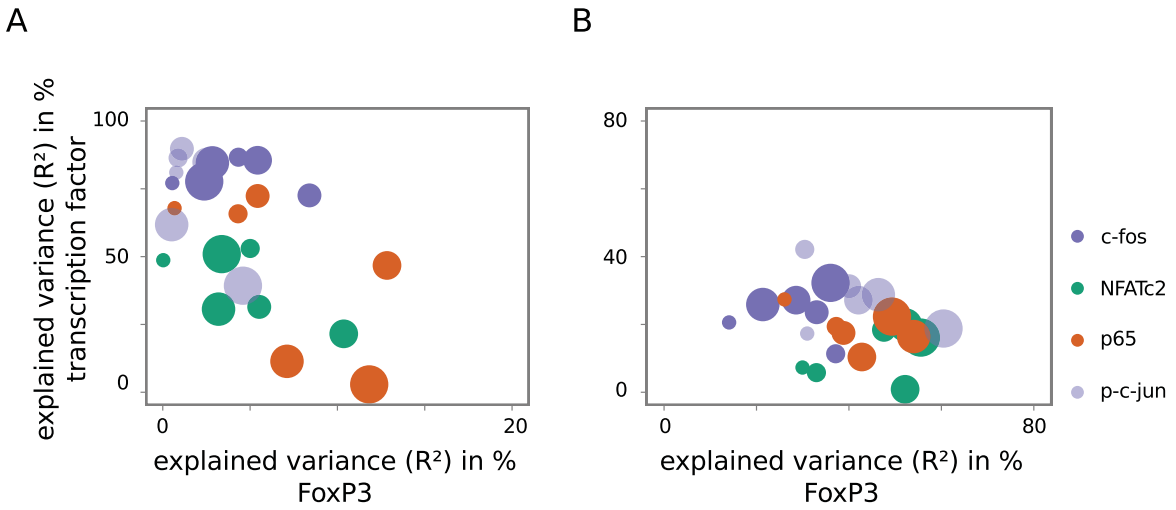

Figure D.1.: Human memory Th cells were stimulated with CD3/CD28. memory T helper cells were harvested at different time-points and co-stained for IL-2, FoxP3, and an additional transcription factor. (A) The dispersion measure for each transcription factor over time in Teff cells is displayed (Table 2.1). (B) The dispersion measure for each transcription factor over time in Treg cells is displayed. Larger points indicate later time-points.

E. Parameter estimation for cooperative and independent transcription factor models

Table E.1.: Parameter estimation for modes of cooperative (Equation 2.14, Section 2.7) and independent (Equation 2.15, Section 2.7) transcription factor models in Teff cells. Parameters and statistical analysis of two models describing the dependency of IL-2 production on transcription factor concentration of c-fos and NFATc2. The model reproducing the experimental data (Figure 3.10) best is indicated by its goodness of fit (GoF) and Akaike's information criterion, corrected for sample size (AICc) and highlighted in red.

model	parameter			goodness of fit measures	
	$a1$	$a2$	b	GoF	$AICc$
independent	0.66	1.52	−0.90	0.0402	−247.43
cooperative	1.71		−0.24	0.035	−258.68

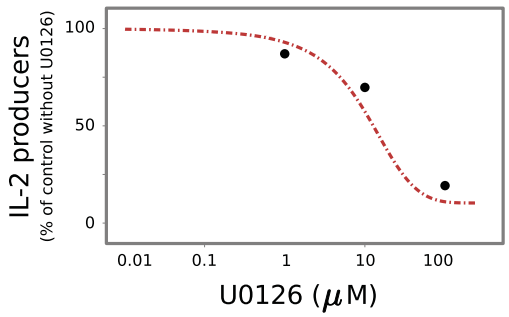

Figure E.1.: Inhibition of c-fos gene expression decreases percentage of IL-2 producers in memory Th cells. Human memory Th cells were preincubated with different concentrations of U0126 for 20 min and subsequently stimulated with PMA/Ionomycin for 5 h. The model prediction of the percentage of IL-2 producers as function of U0126 inhibitor concentration is shown as red line. The percentage of IL-2 producers in dependency of different concentrations of U0126 from the inhibition experiment are shown as black dots.

F. Parameter estimation for inhibitory transcription factor models

Table F.1.: Parameter estimation for inhibitory response models in Treg cells cells. Parameters of the inhibitory model describing the dependency of IL-2 production on the relation between the concentration of an activating transcription factor and FoxP3.

transcription factor	parameter				
	a	b	c_1	c_2	n
c-fos	1.00	0.00	0.00	1.04	4.015
NFATc2	1.00	0.0003	0.00	1.67	3.33
p65	1.00	0.00	0.00	1.91	2.28
p-c-jun	0.00	0.00	0.00	2.30	2.65

List of Publications and Talks

Publications

M. Benary, H. Bendfeldt, R. Baumgrass, and H. Herzel. "Modeling IL-2 gene expression in human regulatory T cells." *Genome Inform*, 20:222-30, 2008

Y. Lee, M. Benary, R. Baumgrass, and H. Herzel. "Prediction of regulatory transcription factors in T helper cell differentiation and maintenance." *Genome Inform*, 22:84-94, 2010

H. Bendfeldt, M. Benary, T. Scheel, S. Frischbutter, A. Abajyan, A. Radbruch, H. Herzel, and R. Baumgrass. "Stable IL-2 decision making by endogenous c-fos amounts in peripheral memory Th cells." *Journal of Biological Chemistry*, 2012

H. Bendfeldt, M. Benary, T. Scheel, K. Steinbrink, A. Radbruch, H. Herzel, and R. Baumgrass. "IL-2 Expression in Activated Human Memory FOXP3(+) Cells Critically Depends on the Cellular Levels of FOXP3 as Well as of Four Transcription Factors of T Cell Activation." *Front Immunol*, 3:264, 2012

R. Lehmann, R. Machne, J. Georg, M. Benary, I. M. Axmann, and R. Steuer. "How cyanobacteria pose new problems to old methods: challenges in microarray time series analysis." *BMC Bioinformatics*, 14:133, 203

in preparation

Yue-Hien Lee, Robert Lehmann, Stefan Kröger, Manuela Benary, Hanspeter Herzel. "cobindR: an R package for analyzing co-occurring transcription factor binding sites"

Diploma thesis

"Modeling the autoregulation of the transcription factor NFATc1", Institut for Theoretical Biophysics, HU Berlin

List of software developments

Y. Lee, R.Lehmann, S. Kröger, M. Benary, `cobindR`, *Bioconductor.org*, 2013

Talks

M. Benary, Modeling of Interleukin-2 Gene Regulation using Flow Cytometry Data, *Invited Talk*, Aberdeen, 2010

M. Benary, Modeling gene expression - A case study using IL-2 gene expression, *IBSB conference*, Berlin, 2011

Berlin, den 29.09.2014 Manuela Benary